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Title: Modified Limiting Dilution Analysis: A Mathematical Model With Biological Interpretation.

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Mario E. Magaña

A mathematical model of Limiting Dilution Analysis for two limiting parameters is presented and investigated. Limiting Dilution Analysis is a microbiological cell assay developed for immunological application. In the given case we deal with the interaction between B lymphocytes, macrophage derived factor and T-independent antigens. The state of the art is that quantitative statements are only possible if one cell type (in general the B cells) is limiting and all others are in excess present.

The basis for this thesis is a set of experiments in which B cells and macrophage derived factor are limiting and all other involved cells and factors are in saturating amounts present. It is shown that so far presented suggestions on modeling Limiting Dilution Analysis for two limiting cell-types are not suitable for this problem. Further, a mathematical model based on data is presented and interpreted in immunological terms with the help of a set of

partial differential equations. The basis for the interpretation of the model are changes in affinity and saturation effects, both not incorporated in the so far presented models of the assay. In particular the relevance of mathematical interpretation of this process for the identification of new concepts as the saturation effects is stressed.

The model of partial differential equations is highly non-linear but offers the possibility of interpreting the highly interrelated processes apart from each other.

Modified Limiting Dilution Analysis: A Mathematical Model With Biological Interpretation

by

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MODIFIED LIMITING DILUTION ANALYSIS: A MATHEMATICAL MODEL WITH BIOLOGICAL INTERPRETATION

CHAPTER 1. INTRODUCTION

Limiting Dilution Analysis (LDA) is a microbiological experiment designed for *in vitro* analysis of cells from the immune system. In its present form it is already well understood and frequently used. This thesis deals with a modified version of this assay, in particular it deals with Limiting Dilution Analysis for two limiting parameters. Traditional LDA has only one limiting parameter.

The assays which are the basis of this thesis deal with T-cell-independent antigen. Macrophage derived supernatant is the second limiting parameter which is added in varying amounts.

In chapters 2 and 3 we present a brief summary of immunology and LDA, both of which are necessary to solve our problem. Chapter 4 presents the data and its description. Then in chapters 5 and 6 we present a mathematical model for the data as well as its biological interpretation. This makes it possible to describe the occurring effects quantitatively and qualitatively. Chapter 7 finally gives a perspective on the possible application of the model.

Special emphasis is made on the interpretation of the model. On the one hand, we present a model which is in a closed form rather than a series expention. This makes interpretation possible and practical. On the other hand, the interpretation is furthered by considering the partial derivatives of

such an equation. This approach is in some way the reverse of what is normally done. We derive a differential equation from a non-differential one, rather than finding a solution of a differential equation. However, this proved to be a very effective way of aiding the interpretation of our model.

CHAPTER 2. IMMUNOLOGY

The following cannot be more than a brief introduction to immunology. For an excellent treatment of the topic see [1, Benjamini et.al.] or other references like [2, 3, 4, 5].

The immune system has the task to defend an organism against foreign intruders which are possibly harmful to it. In fact the very essence of the immune system is this ability to distinguish between the cells or substances which belong to the body and those which are foreign. Thus, the immune system can distinguish between "self" and "non-self". Those intruders, which are called antigen (Ag), are microorganisms like bacteria or viruses; they may be foods, chemicals, drugs, pollen, or animal hair as well. In general it can be any kind of macromolecule.

The immune system possesses numerous ways to fight those contaminants. These possible responses can be grouped in two categories: the innate or non-specific and the acquired or specific immunity.

The term innate immunity describes all those elements of the immune system as the skin, the mucous membranes, the cough reflex, or pH-barriers. Also numerous internal elements are part of the innate immunity, like interferon and other substances released by leukocytes, the enzyme lysozyme, polyamines and phagocytic cells (cells which can "destroy" intruder cells) like granulocytes or macrophages. Macrophages will play an important role in the following discussions, not because of their ability for phagocytosis but because of their interactions with the cells involved in the acquired immunity. However, note, that macrophages react on antigen in a non-specific way.

The acquired immunity on the other hand is highly specific. On contact with a certain antigen, the body mounts an immune response, which is a highly interrelated chain of events. On initial contact with an antigen, the body is able to mount an immune response, however, it is significantly improved on subsequent contacts – hence the term acquired immunity. Thus, the main features of this part of the immune response are its specificity (it can distinguish among different molecular entities), its discrimination between "self" and "non-self", and its memory (improved immune response on second contact). In addition its adaptiveness (ability to respond to previously unknown molecules) is also a general feature.

There are mainly three types of cells involved in the acquired immune response: B lymphocytes (B cells), T lymphocytes (T cells) and macrophages (Mϕ). There are two major components of the acquired immune response: cellular immunity (cells interact to "destroy" the antigen) and humoral immunity (antibody involvement).

The macrophages trap, process and present antigen or secret factors like interleukin 1 (IL-1) on contact with antigen. T cells participate in the cellular immunity and have helping or suppressing effects on B cells. These lymphocytes, however, synthesize and secrete proteins called antibodies (Ab) or immunoglobulins (Ig).

In general, the humoral response needs help from T cells – which actually do not produce antibodies at all. Just for certain T-independent (TI) antigens the help of T cells for the B cells is not necessary. Since the antigens used in the assays for this paper are T-independent the further discussion of the immune system will focus on the B cells on contact with such T-independent antigens.

A B cell has on its surface receptors (surface immunoglobulins, sIg) with

a specificity for a certain antigen. These receptors recognize complex three-dimensional structures (which are parts of antigens) with a certain affinity for this structure. To stimulate a B cell to antibody-production, antigen has to bind to the B cell and in addition accessory functions of macrophages are necessary. No additional help from T cells is needed for T-independent antigens. These accessory functions of macrophages may be presenting the antigen to a B cell or just secreting certain factors (as shown in [7]). The stimulation, proliferation and maturation of B cells into cells which actually produce antibodies is one of the most important parts of this whole chain of events. It will be discussed in more detail at the end of this chapter.

Despite the surface-receptors of a B cell are specific for a certain antigen, there are still a number of other antigens which also react with this B cell surface receptor, but with a lower affinity. Since the antigen will bind first to those surface immunoglobulins with high affinity, the B cells which have surface receptors of high affinity for a particular antigen will be stimulated first, later other B cells with lower affinity. On the other hand the influence of macrophages is also essential for stimulation. Therefore, if there are higher amounts of macrophages (or factor produced by macrophages) present, it is more likely that also B cells with a relatively low affinity become stimulated.

After stimulation, the B cells (which before proliferation and maturation are also called precursor cells) proliferate and differentiate into antibody-producing cells (or plaque-forming cells, PFC). The produced antibodies have the same specificity (or high affinity for a certain antigen) than the precursor cell surface receptors. These antibodies or immunoglobulins have roughly the shape of a "Y", with two variable regions at the "arms" of the "Y", which are of a certain affinity for a part of an antigen; and a constant region. These parts of antigens are called epitopes. Most antigens have a lot of different

epitopes but some have only one type. On contact with such antigens the immunoglobulins bind to two epitopes, possibly on different antigens, what results in precipitation, agglutination, neutralization of toxins, or other possible effects.

Since in this particular experiment of Limiting Dilution Analysis we deal with piscine (= fish) lymphocytes and macrophages, some differences between fish and mammalien systems – especially humans – should be mentioned.

Fish have lymphocytes with B and T functions, as well as macrophages, just like mammals do. Those cells of lymphocytic morphology can be found in spleen, thymus, gut, kidney and peripheral blood. The latter was mainly used for the Limiting Dilution assays.

Since the number of researchers working on the piscine immune system is small and the number of different species of fish, however, is vast, there are still many questions open, which are already solved for mammalien systems. Such are, i.e., the place where the B cells originate (or to be more exact: the bone marrow equivalent), or the different classes of constant regions of antibodies (isotypes) and the switching between different isotypes. Also the way immunological memory works is considerably different to the mammalien way. For an excellent treatment of this topic see [6, Kaattari].

In this thesis, however, the stress lies on the B cell response on a T-independent antigen, which appears to be very similar, if not virtually identical, to the response in mammalian systems. It is shown that in mammals TI antigens need just IL-1 as accessory functions from macrophages [8]. The same is implied for fish by [7] or [9, 10].

The following is a more detailed description of stimulation and differentiation of B cells on contact with T-independent antigen. It is depicted in fig. 2.1. On contact with a T-independent antigen like Lipopolysaccharide

(LPS), the B cell precursor becomes activated to a certain stage in which it needs the help of macrophages in form of the an factor, possibly IL-1 (see [7, 6]). This activated state is characterized through the ability of the B cell to receive the factor, what is shown in fig. 2.1 diagrammatically as IL-1 receptors. On contact with the factor the B cell finally differentiates into a mature plaque forming cell (PFC). In fig. 2.1 the macrophages are referred to as "MAC".

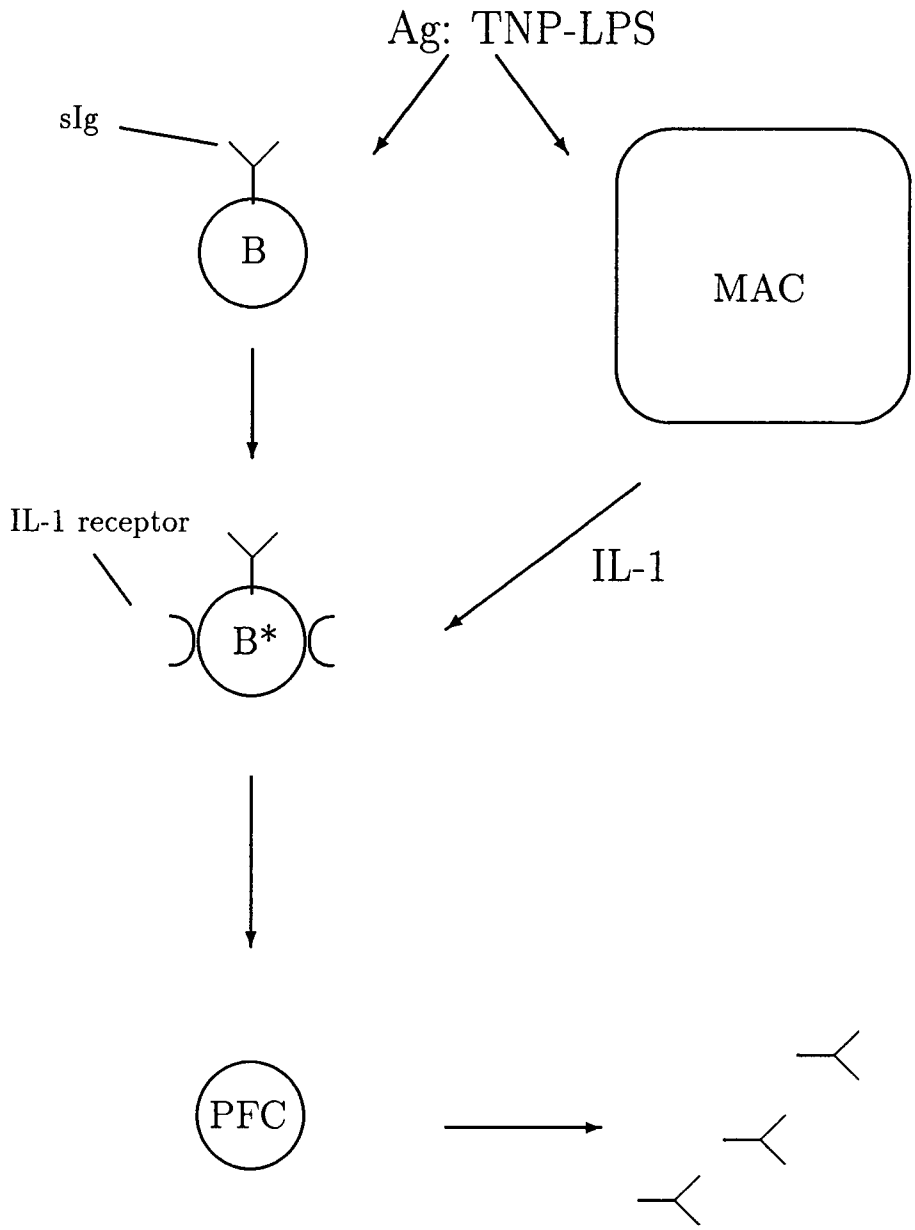

Figure 2.1: B cell interaction, adapted after [6]

CHAPTER 3. LIMITING DILUTION ANALYSIS

3.1 The Experiment

Limiting Dilution Analysis (LDA) is a microculture assay originally developed for one limiting class of cells. It was developed by Lefkovits [11, 12, 1972 and 1979] for the application in immunology. For a good and comprehensive treatment see in particular [12, Lefkovits and Waldmann, 1979]. Briefly it is an assay with all cells of immune system in saturating amounts but one. In this particular example we have B cells as the limiting cell type, macrophages and T-independent antigen in saturating amounts. That means, there is a (high) number of culture wells with macrophages, antigen and other cells of the immune system in excess (= saturating amounts). In these wells a certain amount (or concentration, not saturating) of lymphocytes (B cells) is introduced. For different concentrations of B cells different sets of cultures (all with the same B cell concentration) are investigated. Important is just if a culture responded to the antigen or not. The fraction of non-responding cultures is the basis for the mathematical interpretation of the assay (as described in sec. 3.2).

The new step in the data for this thesis is to make another cell type limiting and investigate the results of that. Macrophages or in this case macrophage derived supernatant is the second limiting cell type of those experiments.

Limiting Dilution Analysis finds wide applications in mammalien systems. In fish, however, the first experiments of this kind were done by Tripp [13, 1989]. The data for this thesis was provided by Mr. Henry Ortega,

M.S., in the Laboratory of Prof. Steven Kaattari, Oregon State University, Corvallis, Oregon. The animal used was rainbow trout. The experiments briefly described in the following are treated in depth in [7, Ortega et.al.]. The leukocytes (macrophages and lymphocytes) were extracted from peripheral blood (PB) and as an antigen, the T-independent trinitrophenylated-lipopolysaccharide (TNP-LPS) was used. Lymphocytes and macrophages were separated through plastic adherence. The adherent cells (macrophages) were washed three times to remove all residual non-adherent cells (lymphocytes). The supernatant was gained through culturing PB leukocytes with TNP-LPS. It was separated from cells and debris by centrifuging. The number of PFC was assayed by Cunningham Plaque Assay.

Limiting Dilution Analysis was performed like previously described [14, 15]. Briefly, lymphocytes were diluted to appropriate concentrations with irradiated filler cells (no antibody production), to ensure a constant concentration of $2 \cdot 10^7$ cells/ml. For each concentration of lymphocytes, 60 replicate culture wells were assayed. Supernatant was added to all cultures in different concentrations.

3.2 Mathematical Interpretation of the Assay

This is meant to be a brief treatment of the mathematical interpretation of the Limiting Dilution Analysis as given by I. Lefkovits and H. Waldman in [12, 1979].

3.2.1 General Treatment

3.2.1.1 The General Poisson Distribution

The basis for Limiting Dilution Analysis is the Poisson distribution, that is

$$F_r = \frac{u^r}{r!}e^{-u}. \tag{3.1}$$

It gives the probability of exactly r "hits" out of a very large possible number c. The probability for a "hit" has to be very low, e.g., $\frac{1}{w}$. The only thing which has to be known is $u = \frac{c}{w}$. This is very special about the Poisson distribution, that the parameter u is the mean $\mu = \frac{c}{w}$ of the distribution (it is actually also the variance).

$$\begin{aligned}\mu = \sum_{r=0}^{\infty} r \cdot F_r &= e^{-u} \cdot (u + 2\frac{u^2}{2!} + 3\frac{u^3}{3!} + \ldots) \\ &= ue^{-u} \cdot \overbrace{(1 + u + \frac{u^2}{2!} + \ldots)}^{e^{+u}} = u.\end{aligned}$$

That means, u is actually the mean number of "hits" (which is $\frac{c}{w}$).

This probability distribution is nothing else than the limit of the binomial distribution for $c \to \infty, w \to \infty$.

3.2.1.2 Poisson Distribution Applied to Limiting Dilution Analysis

In our case we have a high number of lymphocytes c with high affinity for TNP-LPS. If the number of culture wells is w, then $\frac{1}{w}$ is the probability that there is one of those c cells in one particular well.

A "hit" would than mean that in one particular culture well a B lymphocyte precursor specific for TNP-LPS and the antigen TNP-LPS encounter

(with the result of antibody production). A culture well with no "hit" ($r = 0$) is therefore one that doesn't respond to the antigen at all.

Since u is the mean of the probability distribution it is the mean number of "hits" per well. Because the antigen is in excess in every well, it is assumed that if a precursor cell with the right specificity is in a well it does automatically also encounter the antigen. Therefore, u is actually the mean number of precursor cells with high affinity for TNP-LPS per well ($u = \frac{c}{w}$), as it is supposed to be for the Poisson distribution.

In summary we see that the requirements for the possibility of applying the Poisson distribution to the problem of distributing precursor cells in culture wells are a enough high number of lymphocytes with high affinity for TNP-LPS and a high number of wells containing TNP-LPS in excess. All three conditions are in this case fulfilled.

3.2.1.3 Limitations of this Approach

Obviously the big limitation of this approach of Lefkovits et.al. and of applying the Poisson distribution at all (since this problem is inherent to this probability distribution) is that there are some B cell precursors which produce a "hit" and all the others do not in any case. In other words, it is assumed that there are just two kinds of affinities for this antigen: either high affinity (antibodies are specific for this antigen) or no affinity. This is obviously not the case in reality. It seems, however, to be in many cases a valid simplification, especially if just one cell type is limiting (which is the main goal of the original assay). In this thesis, however, the stress is on two limiting cell types. Here it seems that new explanations are necessary.

3.2.2 More Quantitative Treatment

3.2.2.1 Single-Hit Kinetics

If we express the previous statements quantitatively, we see that, if just one cell type is limiting, we can just apply the Poisson distribution as given in eq. 3.1 and get for the number of non-responders:

$$\begin{aligned} r = 0 \quad &\ldots \quad \text{non} - \text{responder} \\ F_0 \quad &= \quad e^{-u} \\ u \quad &= \quad -\ln F_0 \end{aligned} \tag{3.2}$$

Thus, single-hit events yield a straight line in a semi-logarithmic plot as shown in fig. 3.1.

3.2.2.2 Multi-Hit Kinetics

The term multi-hit kinetics is often applied to all assays where more than just one cell is needed to result in antibody production (a "hit"). Originally Lefkovits et.al. distinguishes between multi-hit and multi-target kinetics (see next section) and gives the term multi-hit the meaning that there are at least n cells of one category of cells necessary to result in a reaction. Again the differences in affinity of the B cells for the antigen are not taken into account.

Under these assumptions the probability for not-responding would be a summation of all those probabilities for exactly k hits with $k < n$.

F_k	e^{-u}	ue^{-u}	$\frac{u^2}{2!}e^{-u}$	$\ldots$	$\frac{u^{n-1}}{(n-1)!}e^{-u}$
k	0	1	2		$n-1$

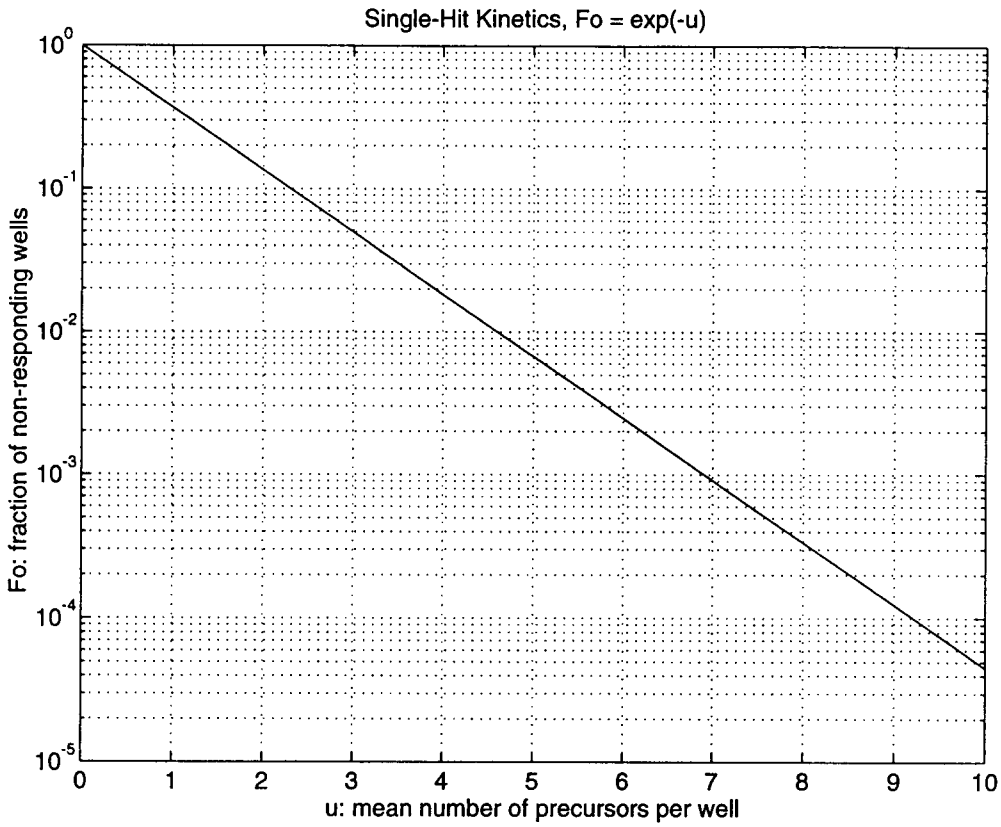

Figure 3.1: Single Hit Kinetics of Limiting Dilution Analysis

Thus, the probability for non-responding in this model is

$$F_0 = \sum_{k=0}^{n-1} \frac{u^k}{k!} e^{-u} \tag{3.3}$$

This probability is given in fig. 3.2 for several n. Note that these curves neither assume the same slopes for high u nor do they have a defined, but for each n different, slope in the first portion of the curve (small u).

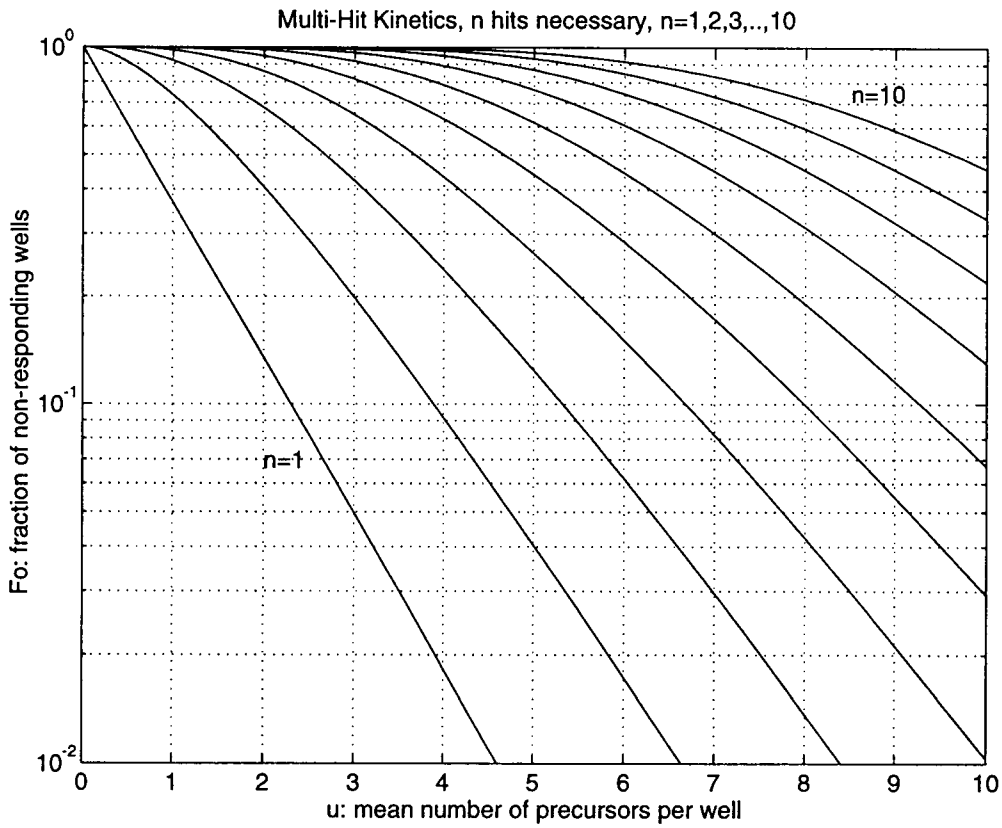

Figure 3.2: Multi-Hit Kinetics of Limiting Dilution Analysis

3.2.2.3 Multi-Target Kinetics

A multi-target event takes place if there is one cell of each of m different cell classes necessary to invoke an antibody response. In terms of the probabilities of the Poisson distribution, this would mean:

$$1 - e^{-u} = \text{probability for a well to contain one or more cells of one category}$$

$$(1 - e^{-u})^m = \text{probability for a well to contain one or more cells of } m \text{ categories}$$

Therefore the fraction of non-responding culture wells is defined by

$$F_0 = 1 - (1 - e^{-u})^m \quad \ldots \quad \text{probability for a well to miss at least one of the } m \text{ categories}$$

This probability is shown in a semi-logarithmic plot in fig. 3.3. As can be seen from the plot, this family of functions has a well defined slope for large u which equals the slope the curve for $m = 1$. It is, however, impossible to distinguish a well defined slope for the first portion of these graphs. It is again well worthwhile to note the limitations of this approach. The differences in affinity are again not taken into account. In addition, these formulas are correct (even within the limitations of the Poisson-distribution-approach) only if the size of each category is the same, that means the ratio of sizes is 1:1.

If we have different sizes of the cell populations this can be taken into account. To take the example of a variable ratio of size of macrophage and B cell populations, we can state the following equations:

$$\begin{aligned} F_0 &= 1 - (1 - e^{-u_B})(1 - e^{-u_{M\phi}}) \\ &= e^{-u_B} + e^{-u_{M\phi}} - e^{-(u_B + u_{M\phi})} \\ \text{with} \quad & a = \frac{u_{M\phi}}{u_B} \\ F_0 &= e^{-u_B} + e^{-au_B} - e^{-(a+1)u_B} \end{aligned} \tag{3.4}$$

This probability is given in fig. 3.4 for some ratios $a = u_{M\phi} : u_B$.

3.2.2.4 Frequency Estimation

One of the main goals of this assay is to determine how many B cell precursors with high affinity (or specificity) are in the total pool of lymphocytes,

or what the ratio or frequency of precursors per lymphocytes is. To make this more transparent, we will introduce two new variables, C and U. Thus, we know the following:

c ... total number of precursors specific for antigen

C... total number of lymphocytes (specific or non-specific)

w... number of culture wells

u ... number of precursors per well, $u = \frac{c}{w}$

U... number of lymphocytes (specific and non-specific) per well, $U = \frac{C}{w}$

The frequency is therefore:

$$f = \frac{\text{number of specific precursors}}{\text{total number of lymphocytes}} = \frac{c}{C} = \frac{u}{U} \tag{3.5}$$

The values of C, U are known, u or c are the goal of calculation and can for single-hit kinetics easily calculated by the Lefkovits-formula eq. 3.2.

3.2.2.5 Graphs obtained through Limiting Dilution Analysis

Since through Limiting Dilution Analysis the number of specific precursors per well u or the precursor frequency $f = \frac{u}{U}$ is to be determined, it is impossible to graph F_0 with u. Therefore normally F_0 with U is plotted. It is now assumed that the precursor frequency $f = \frac{u}{U} = \frac{c}{C}$ does not change with increased cell input. Than, since

$$U = \frac{1}{f} \cdot u$$

For a constant frequency f, U is linearly related to u.

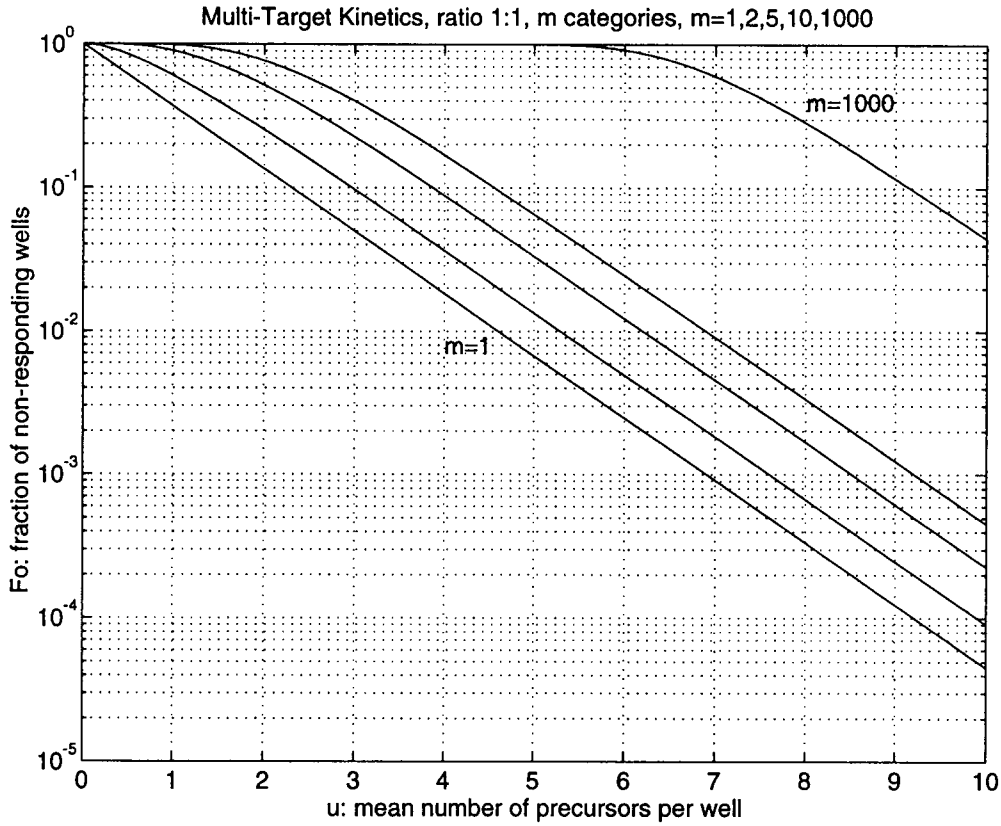

Figure 3.3: Multi-Target Kinetics of Limiting Dilution Analysis (all cell categories have the same size)

For $f = const$ it is, thus, valid to use a graph F_0 with U to determine what kind of relationship this particular assay represents (i.e. linear relationship which means single-hit kinetics), to calculate u on the basis of this knowledge and than to find the frequency f (which actually would enable us to transform U into u). In our case, however, the frequency might depend on U. Than $f \neq const$ would be the case, and we get the general equation

$$u = f(U) \cdot U. \tag{3.6}$$

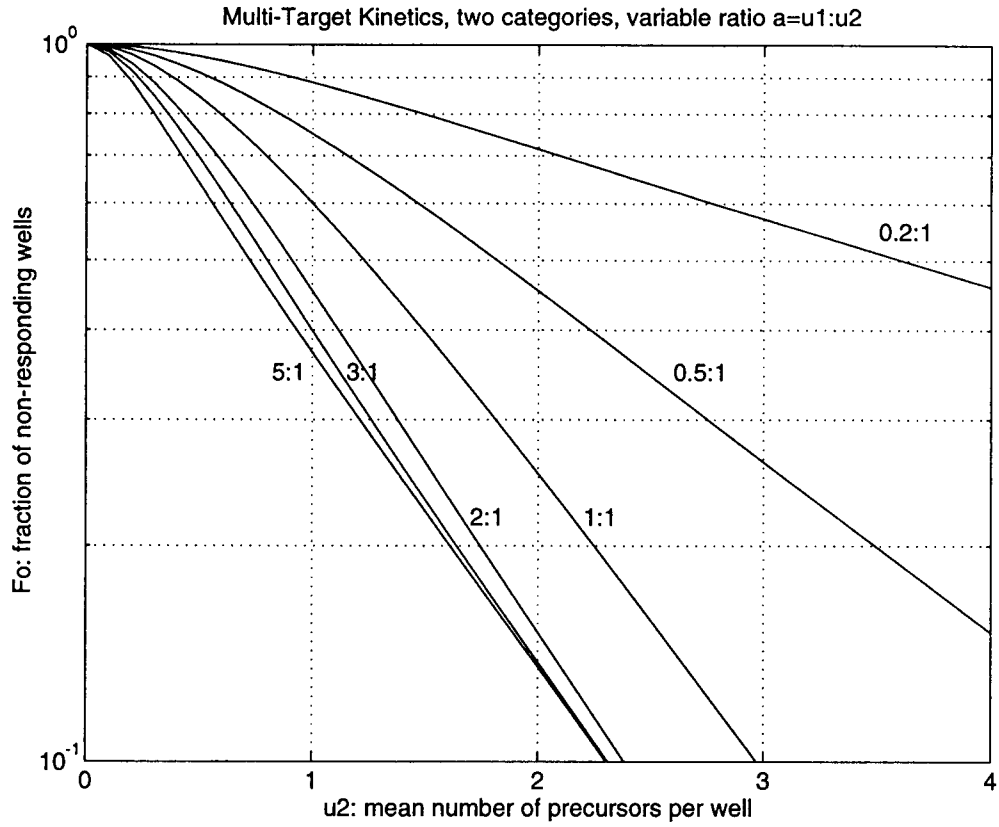

Figure 3.4: Multi-Target Kinetics of Limiting Dilution Analysis (variable ratios $a = \frac{u_1}{u_2}$)

Chapter 4. The Data

One of the most crucial aspects in finding a model is to have accurate enough data to validate it and to find the parameters that describe it. In what follows, the data which is the basis for our model is described.

4.1 Reliability of the Data

The reliability of the data deserves some consideration. Every set of data was obtained from the peripheral blood of one fish. Therefore there is a limited maximal number of culture wells. Thus, we have a trade-off between number of data points and number of culture wells assayed per data point. If we want a high number of data points for the curves, we cannot use many culture wells to validate those points. For this reason we stick with the convention proposed by Lefkovits [12], which is, one should use at least 60 samples per data point. All data plots described in this chapter are based on 60 culture wells per data point.

In appendix A the complete numeric data is given. There also confidence intervals as adapted from Lefkovits [12] are given. These confidence intervals show in which interval the actual fraction of non-responding culture wells lies with 95 % probability. However, the given confidence intervals are only for excess of supernatant the real confidence intervals, since they are calculated for a Poisson-distributed F_0. For limiting amounts of supernatant, we would have to know the probability distribution of F_0. However, this is initially unknown, since the equation for F_0 is initially unknown. Thus, these confidence intervals are merely a convention.

The confidence intervals in appendix A suggest that the data points are more reliable for higher fraction of non-responding culture wells, especially because of the semi-logarithmic plot.

In considering the reliability of the data, we also have to note that all the data sets fulfill the specifications of the next sections and sec. 5.2.1 uniformly very well.

Concluding, we may say, that the data should not be used to validate details of the mathematical description, but it gives enough certainty to formulate specifications (or requirements on the model) as given in the following sections or sec. 5.2.1.

4.2 Description of the Graphs

As can be seen in figs. 4.1, 4.2, 4.3, and 4.4, the graphs contain three distinct parts: A nearly linear part for small values of cell input, a transition area (the "knee"), and another nearly linear part for high values of cell input.

4.2.1 Different Slopes at the Beginning

The experiments depicted in figs. 4.1, 4.2 already suggest that there are, for small U, different but well defined slopes in the data plots. The experiment shown in fig. 4.4 is specifically designed to investigate the initial slopes in this type of LDA. Therefore, only values of U from 0 to $5 \cdot 10^4$ are depicted, instead of $10 \cdot 10^4$, as in the other graphs. For this reason this graph does not show the effect of the "knee." This graph together with the other data clearly suggests that there are distinct and well defined slopes for each concentration of supernatant in the beginning portion of the data.

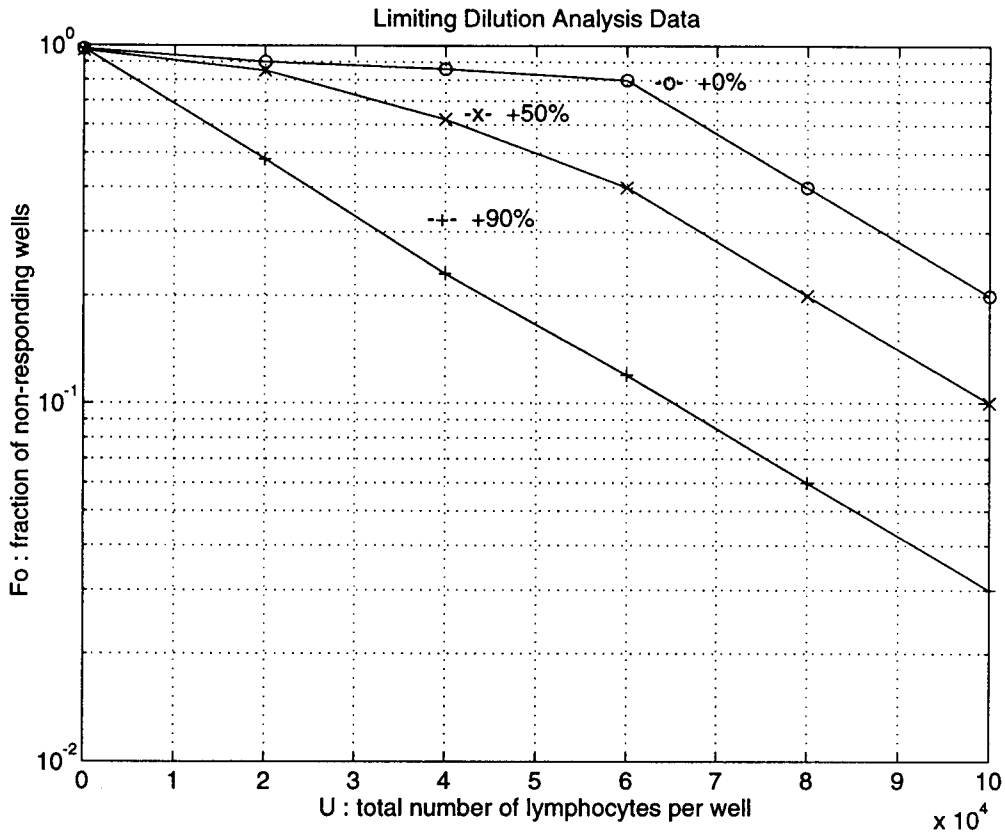

Figure 4.1: Data of Limiting Dilution Analysis with different concentrations of factor

4.2.2 The "Knee"

With the term "knee" we refer to the portion of the data plots where the slope of the graph changes drastically. The experiment shown in fig. 4.3 is especially designed to show this change in slopes, but it is also obvious in figs. 4.1, 4.2. The "knee" is well defined for low concentrations of supernatant, because in this case there is a big difference between the initial and the final slope. For higher concentrations of supernatant, on the other hand, the knee

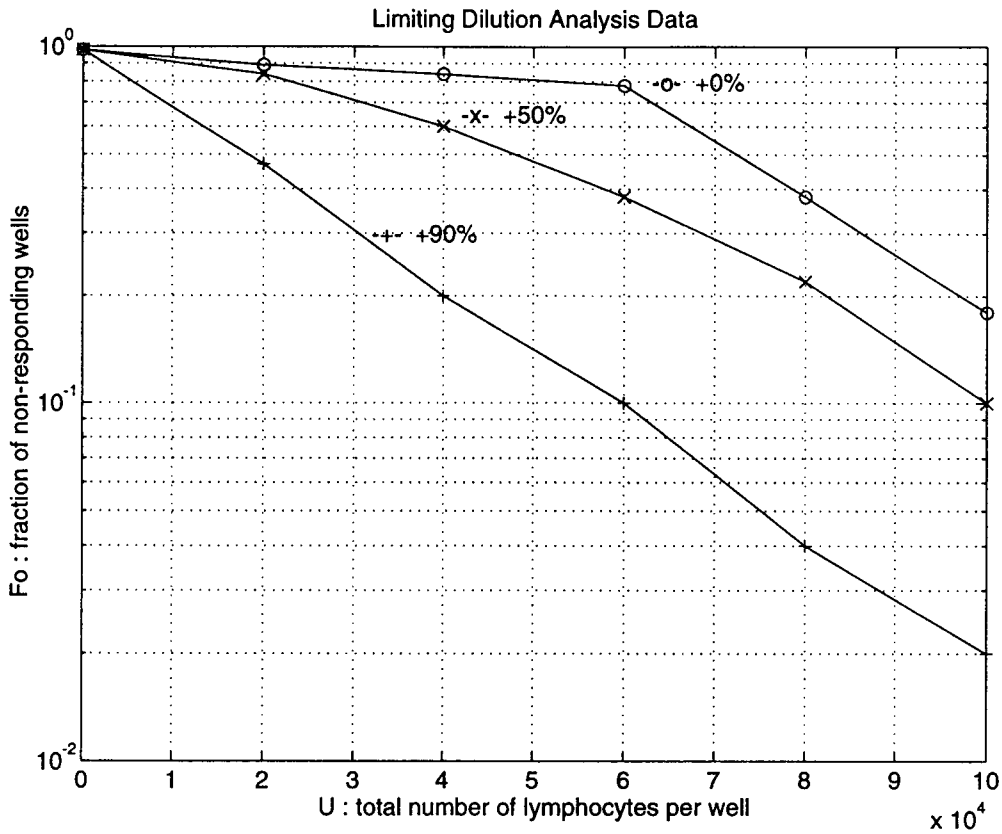

Figure 4.2: Data of Limiting Dilution Analysis with different concentrations of factor

is not so obvious.

The whole phenomena of a "knee" is well known from Bode-diagrams in Control Engineering. This will be discussed in sec. 5.3 and 5.5.2.

4.2.3 Same Slope at the End

As the graphs figs. 4.1, 4.2, 4.3 suggest we will assume (on the basis of the data) that the final slope of the graphs is always the same. That means in particular that it is the same slope as obtained for single-hit kinetics.

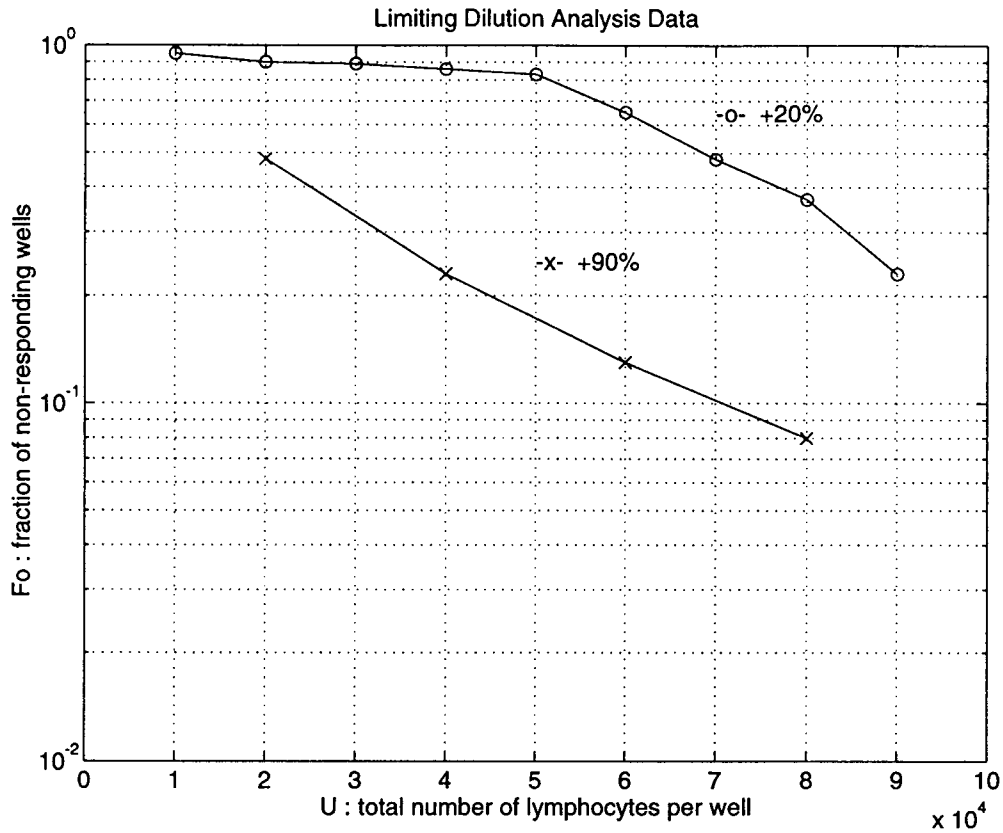

Figure 4.3: Data of Limiting Dilution Analysis with different concentrations of factor

4.3 Multi-Hit or Multi-Target Kinetics Cannot Explain the Graphs

If we compare the given data as described above (sec. 4.2) with the multi-hit or multi-target kinetics as given in sec. 3.2.2.2 or depicted in figs. 3.2, 3.3, and 3.4, it is obvious that these approaches cannot explain the data that we deal with here.

None of these approaches has a resemblance to the first portion of the data graphs, also the constant slope at the end occurs only in multi-target

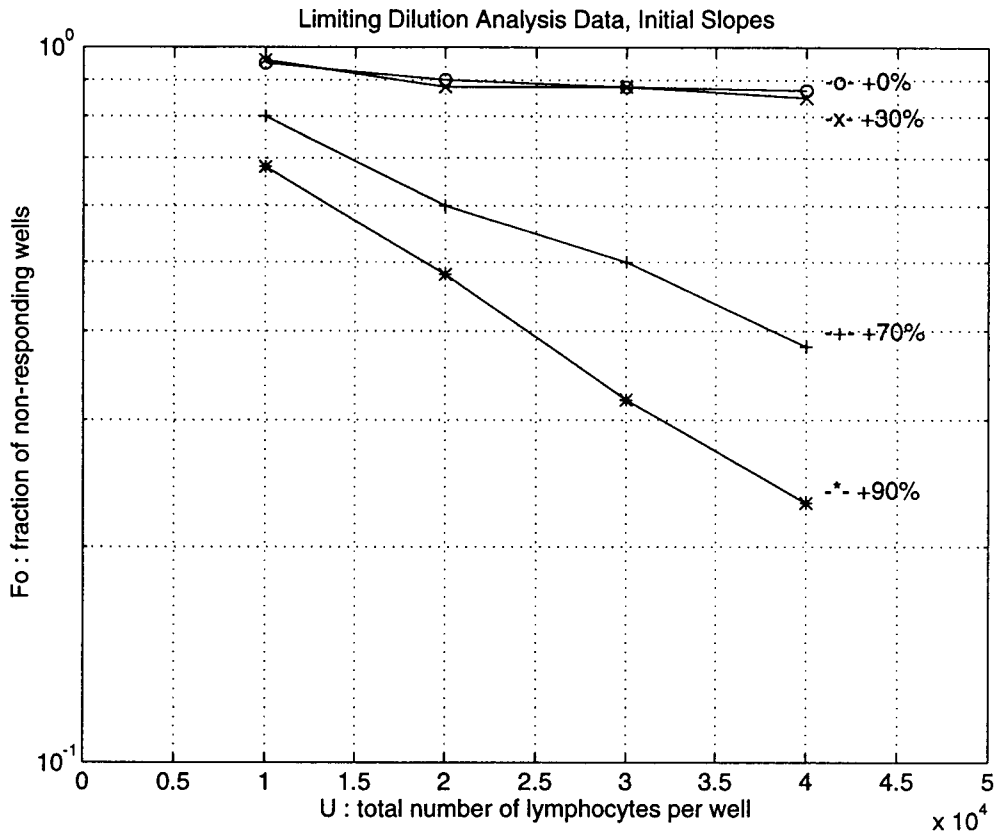

Figure 4.4: Data of Limiting Dilution Analysis with different concentrations of factor

kinetics with many categories and ratios of 1:1.

In our opinion, multi-hit and multi-target kinetics cannot explain the results of this experiment since they do not take into account the change in affinity which occurs if the concentration of macrophage derived factor is varied.

4.4 Saturation Effects

It is possible to distinguish two completely different saturation effects associated with this data. Both of them are related with the concentration of supernatant (macrophage derived factor, Interleukin-1).

4.4.1 Excess of Supernatant

It is expected from biological considerations and is also obvious from the data that there will be a concentration of supernatant beyond which an increase in the amount of supernatant will not change the biological reactions any more.

At that point the supernatant ceases its effects as a parameter and therefore we deal again with single-hit kinetics and should get the same experimental (and theoretical) results as previous researchers (like [12, Lefkovits]).

The result of this saturation effect in the data is that we get a straight line without the "knee" or two different slopes just as in normal single-hit kinetics for 90% of supernatant.

The mathematical model will have to take into account this effect.

4.4.2 Macrophage Contamination

Ideally, none of the culture wells respond if we have no supernatant since no antibody-antigen reaction can take place. However, due to the experimental limitations it is practically impossible to achieve actual 0% supernatant since in a real assay there will always be a small amount of contaminating macrophages in the population of lymphocytes.

Although in theory we expect that the fraction of non-responding wells F_0 to be 1 for all U (horizontal line at $F_0 = 1$), the data for 0% supernatant

and 30% supernatant in fig. 4.4 hardly differ. This effect is due to the contamination with macrophages.

In the theoretical model we want to achieve this horizontal line at $F_0 = 1$ and take into account this contamination (or saturation effect) by acknowledging that the actual concentration of macrophage derived supernatant in the data is not 0% .

4.5 The Horizontal Axis

As mentioned earlier (sec. 3.2.2.4 and 3.2.2.5) we are normally interested in the number of precursors per well specific for the antigen, u, but we know only the total number of lymphocytes (specific or non-specific) per well, U.

All the graphs have U, and not u, as variable. The relationship between the two is

$$u = f \cdot U \text{ (for excess of supernatant)}, \tag{4.1}$$

if f does not depend on U. With f we refer to the frequency of specific precursors in the total amount of cells (it is naturally unknown).

It is safe to assume that the frequency f remains constant over all u and U for excess of supernatant. This assumption is used implicitly in all previous research. However, if supernatant becomes the second limiting parameter, the situation might be different. In general f might depend on U or the supernatant concentration. Therefore, a mathematical model will be crucial to calculate the frequency. We will have to deal with the general equation $U = f(U) \cdot U$. This is discussed in depth in sec. 6.4.

CHAPTER 5. MATHEMATICAL STRUCTURE OF THE DATA

5.1 Possibilities for Modeling

We can basically distinguish between mathematical models derived from data and those descriptions derived from physical or biological laws. The latter laws are adaptations of more general laws for a special case, while for the former the laws are unknown. In that case, mathematical laws might be derived from the data. These laws will be specific for the process described by the data. This is our approach in this thesis. The laws we deal with have the form of mathematical equations which have to be filled with biological meaning.

In this chapter, we derive the mathematical equation (or law) associated with our problem. In the next chapter we present the biological meaning of this equation.

Not all models derived from data attempt to formulate "laws" like we do here. Often complicated systems are approximated by a series of terms. Since we want to interpret our results in a biological sense, this approach is useless. Instead of such an approximation we came up with a closed form expression.

A very good introduction to mathematical modeling of biological systems is Gold [16, 1977]. This book covers a range of topics from the basics of modeling (like the modeling process) to parameter estimation, but everything on a relatively low mathematical level. In the beginning of his book the author distinguishes between "correlative" and "explanatory" models. This is rather similar to what we mentioned above. Gold defines "correlative" models as those derived from data. Relations are derived from data and used for

prediction. The data or the derived relations might be used to gain concepts as well (which is the goal of our work). In that case the "correlative" model interacts with an "explanatory" one. An "explanatory" model is one derived from concepts rather than data. The concepts are used to obtain relations and understanding. Data is only necessary for justification of the obtained relations. In contrast to purely "correlative" models, these models can be used for prediction and interpretation, rather than only prediction.

In our case, we have a "correlative" model in the sense that we start from data rather than concepts, but we gain relations from the data which can be used to find concepts. Therefore it is possible to interpret the results like in an "explanatory" model.

It might be interesting to consider briefly some examples of mathematical modeling in biology and especially in immunology. The following is meant to be an overview of some interesting literature. As we will see, we could not find examples of modeling Limiting Dilution Analysis other than presented by Lefkovits [12].

Murray [17, 1977] gives many examples in a number of biological fields of applications of non-linear differential equation models. However, he does not mention models in immunology. The two volumes edited by Iyengar [18, 1984], [19, 1992] focus on the aspects and problems of applying computers in the modeling process.

Models and other theoretical considerations for immunology in particular are presented in the volumes named "Theoretical Immunology", edited by Bell, Perelson and Pimbley [20, 1978] and Perelson [21, 22, 1988]. Furthermore we referenced [23] - [35], which include papers or books by R. Mohler [23, 24, 26, 27, 28, 29, 34, 35], G. Marchuk [24, 25, 30], C. Bruni [23, 24, 27, 28] or many others. Especially [23, Mohler et.al., 1980] give an introduction to

many of these approaches of mathematical modeling in immunology. In most cases, the humoral immune response is modeled. In particular, the amount of antigens, antibodies or lymphocyte populations is described over time. Non-linear or bilinear ordinary differential equations are applied to fulfill that task. Generally the model was found in the way Gold [16] calls an "explanatory" model. From concepts, like the mass action law or statistical considerations, the ordinary differential equations were derived. Oftentimes, even the parameters of these equations are gained through knowledge of concepts rather than through data due to lack of sufficient data. The biological systems modeled in these examples are in two ways fundamentally different from our experiment.

- In LDA time is in excess present, i.e., time has no influence.
- The same is true for the amount of antigen.

Both, time and amount of antigen, are very important in the above discussed models.

Despite the fact that there are many mathematical models of biological systems presented in the literature, Limiting Dilution Analysis is so far only modeled as presented by Lefkovits [12], thus, this is the first model of Limiting Dilution Analysis for two limiting parameters.

5.2 Structure of the Model

5.2.1 Requirements

The model has to fulfill the requirements stated in sec. 4.2 and sec. 4.4, that is,

- the graphs have to show well defined linear slopes for small values of U, different for each concentration of supernatant,

- there has to be a "knee," a drastic change in slopes,
- for large values of U the graphs for all supernatant concentrations have to assume the same linear slope,
- for an excess of supernatant the graph F_0 with U has to be just a line with well defined slope $\neq 0$, the same as for single-hit kinetics,
- for no supernatant the fraction of non-responding cells F_0 will always be 1,
- the initial condition is $F_0(0, \lambda) = 1$.

Note that the graphs are always the semi-logarithmic plots of the fraction of non-responding culture wells, F_0, with the total amount of lymphocytes per well, U.

5.2.2 Notation

In this chapter, the data graphs are treated as representations of some purely mathematical function. This function has to depend on two variables, which are the amount of lymphocytes and the amount of supernatant.

As in the data graphs, the amount of lymphocytes will be used as an independent variable while the amount of supernatant will be used as an independent parameter.

In the following we will be concerned with functional relationships between F_0 and U, with λ as a parameter, where

F_0 ... fraction of non-responding culture wells, vertical axis
U ... total number of lymphocytes per well, horizontal axis
λ ... measurement for the per capita amount of supernatant per culture well, parameter

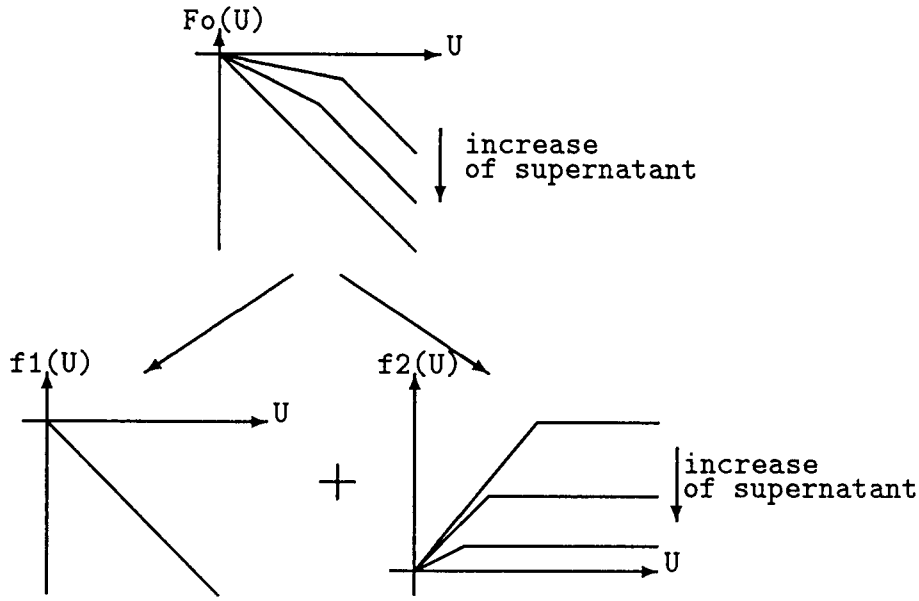

Figure 5.1: Decomposition of the Data Plots

Our approach does not follow the convention used by previous researchers (especially Lefkovits and Waldmann [12]). They presented equations for $F_0(u)$ rather than $F_0(U)$, where u is the number of lymphocytes specific for the antigen. The relation between u and U is given in eq. 4.1 in sec. 4.5 or more specific in eq. 6.5 in sec. 6.4.

In our case the choice of U as variable is necessary since the frequency f cannot be assumed to be constant.

5.3 Decomposition

5.3.1 The Graphs

To make it easier to find a mathematical representation of the graphs, they are first decomposed as depicted in fig. 5.1.

As fig. 5.1 indicates, this decomposition is graphically an additive one: if we add the graphs of `f1(U)` and `f2(U)` we get `Fo(U)` ($= F_0(U)$). However, because this graphs are all semi-logarithmic (with logarithmic vertical axis) this summation is a multiplication in the equation for F_0.

The advantage of this decomposition is that the two graphs are very similar to the Bode diagrams of an Integral Controller and of a Differentiator with a time constant. These similarities are, however, restricted to mathematics, that is, there is no clear connection with elements of control engineering, since the Bode diagram depicts the (in this case) absolute value of the Laplace transform of the integrating or differentiating time-domain equations, and it shows this in a log-log-plot (not semi-logarithmic).

The knowledge of these Bode plots was the key for finding the mathematical model for the process in question (LDA) and an important aid for its interpretation. In addition, the in-depth knowledge of the mechanism and especially the effects of the parameters helps significantly for finding biological explanations.

5.3.2 Associated Equations

The equations associated with the Bode diagrams are:

$$\text{Integrator}: \quad f_I(s) = \frac{1}{s}.$$

$$\text{Differentiator with time constant}: \quad f_{DT_1}(s) = \frac{s}{1+Ts}.$$

The parameter T is associated with the knee, in particular

$$s_{knee} = \frac{1}{T}.$$

For an in-depth treatment of this see [36, Lauber] or [37, Unbehauen].

One can go from a log-log-plot to a semi-log-plot by substituting the variable from the (later) non-logarithmic axis by e to the power of this variable. Thus we get

$$\begin{aligned} f1(U) &= e^{-U}, \\ f2(U) &= \frac{e^{U}}{\sqrt{1+\tau^2 e^{2U}}}. \end{aligned}$$

The square root is due to the fact that we are concerned with the absolute value rather than a complex function.

In the case here we do not just have a simple "differentiator", since this would mean only one slope at the beginning. To achieve different slopes we have to take the function to the power of a parameter

$$\begin{aligned} f1(U) &= e^{-U}, \\ f2(U) &= \left(\frac{e^{U}}{\sqrt{1+\tau^2 e^{2U}}}\right)^{\lambda}. \end{aligned}$$

The function $f1(U)$ is not changed in this step, since the slope is fixed, only $f2(U)$ is effected.

The parameter τ is associated with the location of the "knee" (drastic change in slopes). In particular

$$e^{U_{knee}} = \frac{1}{\tau}, \tag{5.1}$$

$$U_{knee} = -\ln \tau. \tag{5.2}$$

We get together

$$\tilde{\tilde{F}}_0(U,\lambda) = e^{-U} \cdot \left(\frac{e^U}{\sqrt{1+\tau^2 e^{2U}}}\right)^{\lambda}. \tag{5.3}$$

5.4 Initial Condition

Since F_0 is a *probability* or the *fraction* of non-responding culture wells, initially it always has to be 1. If there are no lymphocytes in the well ($U = 0$) then there cannot exist any culture wells which respond to the antigen. Therefore this has to be taken care of in the mathematical equation, that is,

$$\begin{aligned} \tilde{\tilde{F}}_0(0,\lambda) &= e^{-0} \cdot \left(\frac{e^0}{\sqrt{1+\tau^2 e^0}}\right)^{\lambda}, \\ &= \left(\frac{1}{\sqrt{1+\tau^2}}\right)^{\lambda}. \end{aligned}$$

However, as has been stated, the initial condition is:

$$F_0(0,\lambda) = 1. \tag{5.4}$$

Thus, we obtain by taking into account the initial condition

$$\tilde{F}_0(U,\lambda) = \left(\sqrt{1+\tau^2}\right)^{\lambda} \cdot e^{-U} \cdot \left(\frac{e^U}{\sqrt{1+\tau^2 e^{2U}}}\right)^{\lambda}. \tag{5.5}$$

5.5 Parameters

5.5.1 U and u

This section is concerned with the requirement, that for an excess of supernatant the graph has to show the same behavior as for single-hit kinetics (cf. sec. 5.2.1 and 4.4.1).

But the Lefkovits-formula eq. 3.2 (which describes our model for excess of supernatant) is written in terms if u and not U. Thus we have to use eq. 4.1 to achieve a final model of the assay, which says

$$u = f \cdot U \text{ (for excess of supernatant).}$$

As previously mentioned (sec. 4.5 and 5.2.2, cf. sec. 6.4), f can be assumed to be constant for excess of supernatant. Therefore, we introduce the parameter a and get so the final equation for the fraction of non-responding culture wells F_0

$$F_0(U,\lambda) = \left(\sqrt{1+\tau^2}\right)^{\lambda} \cdot e^{-aU} \cdot \left(\frac{e^{aU}}{\sqrt{1+\tau^2 e^{2aU}}}\right)^{\lambda}. \tag{5.6}$$

From the requirement we know that $a = f(\lambda = 0)$ [1]. It is perhaps not immediately obvious, why we have just one parameter a despite the fact that the requirement is only concerned with the term e^{-aU}. However, if we have different parameters in the argument of the exponential function the shape of the graph changes and other requirements are no longer fulfilled. But if the parameter is everywhere the same, it is only a scaling factor with effect on the horizontal axis.

5.5.2 The "Knee"

The "knee" is the portion of the graphs where the two pseudo-linear parts come together, and where the slope of the curve changes drastically. The U-value where this occurs is called U_{knee}; it depends on $\tau(\lambda)$.

The data is unfortunately not very clear about the location of this

[1] On the range of λ see sec. 5.5.3.

portion, which means that the function $\tau(\lambda)$ is not well defined by it.

Despite that, for a complete model it is necessary to model the location of the "knee" as well. Therefore, we will define a function $\tau(\lambda)$ on the basis of expectations on the model.

These expectations are

1. the "knee" will shift with λ; for $\lambda = 1$ it will be at $U_{knee} = \infty$ [2],
2. the values for U_{knee} are relatively close together for a wide range of λ,
3. for $\lambda \to 0$, U_{knee} will approach 0 or $-\infty$.

The reasons for these expectations are

1. this is necessary to fulfill the requirement of $F(U, 0) = 1 \; \forall U$,
2. this follows more or less from the graphs, but it might change with better data on this problem,
3. this seems reasonable, it is however of no importance.

A function which fulfills all these expectations is

$$U_{knee} = k_1 \ln(-k_2 \cdot \ln(1 - \lambda)). \tag{5.7}$$

In fig. 5.2 this function is shown for different values of (k_1, k_2).

The actual values for (k_1, k_2) can be chosen to fit the graphs best.

Now, from eq. 5.1 and considering sec. 5.5.1, we get

$$e^{aU_{knee}} = \frac{1}{\tau}, \tag{5.8}$$

[2] On the range of λ see sec. 5.5.3.

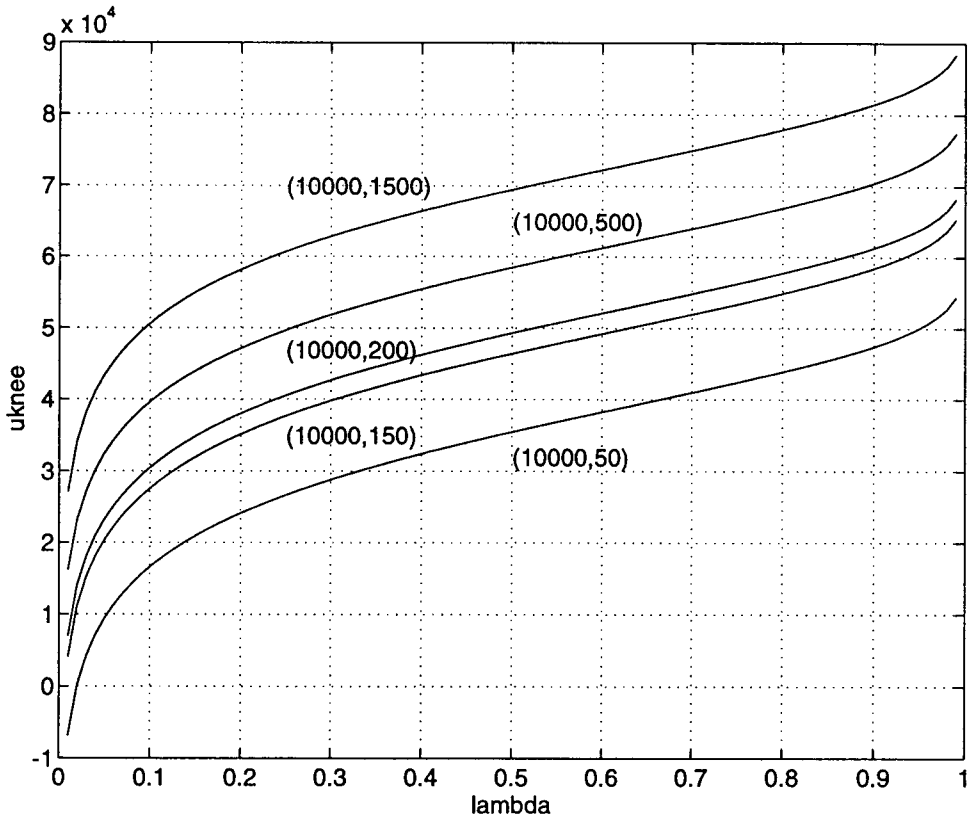

Figure 5.2: Some Possible Knee-Functions for Different Pairs of Parameters (k_1, k_2)

and

$$\tau(\lambda) = [-k_2 \cdot \ln(1-\lambda)]^{-ak_1} . \tag{5.9}$$

5.5.3 Range of λ and F_0

To obtain similar results to the data plots, λ takes on the values

$\lambda = 0$ ... excess of supernatant,

$\lambda = 1$ ... no supernatant.

Therefore, λ is actually a measure for the lack of supernatant.

Furthermore,

$$F_0(U,\lambda) \in [0,1] \quad \forall U \geq 0, \lambda \in [0,1].$$

This is required since F_0 is a fraction or probability.

Proof:

As previously shown (sec. 5.4, eq. 5.4)

$$\lim_{U \to 0} F_0(U,\lambda) = F_0(0,\lambda) = 1.$$

Furthermore

$$\lim_{U \to \infty} F_0(U,\lambda) = \lim_{U \to \infty} \left(\sqrt{1+\tau^2}\right)^{\lambda} \cdot e^{a(\lambda-1)U} \cdot \left(\frac{1}{\sqrt{1+\tau^2 \cdot e^{2aU}}}\right)^{\lambda}.$$

Since

$$\lambda - 1 \leq 0 \quad \forall \lambda \in [0,1],$$

we get

$$\lim_{U \to \infty} F_0(U,\lambda) = 0.$$

in addition, we know

$$F_0(U,\lambda) \geq 0 \quad \forall U \geq 0, \lambda \in [0,1]$$

since all terms in $F_0(U,\lambda)$ are greater or equal to zero (for real τ).

Taking the partial derivative with respect to U, yields

$$\frac{\partial}{\partial U} F_0(U,\lambda) = -aF_0(U,\lambda) + a\lambda F_0(U,\lambda) \cdot \frac{1}{1+\tau^2 e^{2aU}}.$$

It is always

$$\frac{1}{1+\tau^2 e^{2aU}} \leq 1.$$

Therefore, for

$$\lambda \leq 1,$$

we get

$$\frac{\partial}{\partial U} F_0(U,\lambda) \leq 0 \ \forall U \geq 0, \lambda \in [0,1].$$

For a negative partial derivative in U and the given limits, it follows that

$$F_0(U,\lambda) \in [0,1] \ \forall U \geq 0, \lambda \in [0,1]. \quad (q.e.d)$$

5.6 Mathematical Model Derived from Data

Our proposed mathematical model for Limiting Dilution Analysis with two limiting parameters is therefore

$$F_0(U,\lambda) = \left(\sqrt{1+\tau(\lambda)^2}\right)^{\lambda} \cdot e^{-aU} \cdot \left(\frac{e^{aU}}{\sqrt{1+\tau(\lambda)^2 \cdot e^{2aU}}}\right)^{\lambda}, \qquad (5.10)$$

where

$$\tau(\lambda) = [-k_2 \cdot \ln(1-\lambda)]^{-ak_1},$$

with

$$F_0(U,\lambda) \in [0,1] \ \forall U \geq 0, \lambda \in [0,1],$$

and

$$k_1, k_2, U \geq 0, \lambda \in [0,1].$$

This model fulfills all the requirements given in sec. 5.2.1. Furthermore this equation is decomposable and the effects of the parameters on the shape

of graphs are well understood. This makes this model a good starting point for biological interpretation.

The graph in fig. 5.3 depicts the model as given in eq. 5.10.

The four graphs in fig. 5.4 depict some mathematical models for variations of the parameters, in particular for small and large k_2 (which shifts U_{knee}, and small and large a (which changes the slopes of all curves uniformly).

Note that the choice of a function for $\tau(\lambda)$ is so far rather arbitrary, since we don not have sufficient data to validate it. However, the choice of $\tau(\lambda)$ does not affect the model as proposed in eq. 5.10.

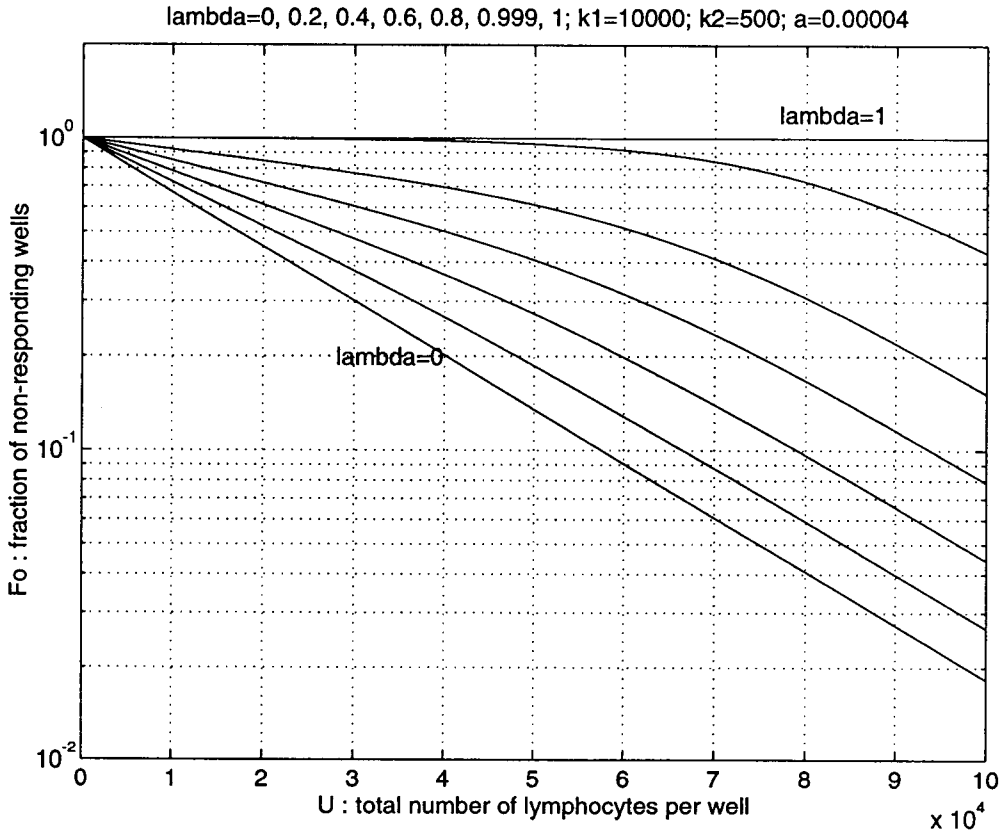

Figure 5.3: The Mathematical Model for the Data

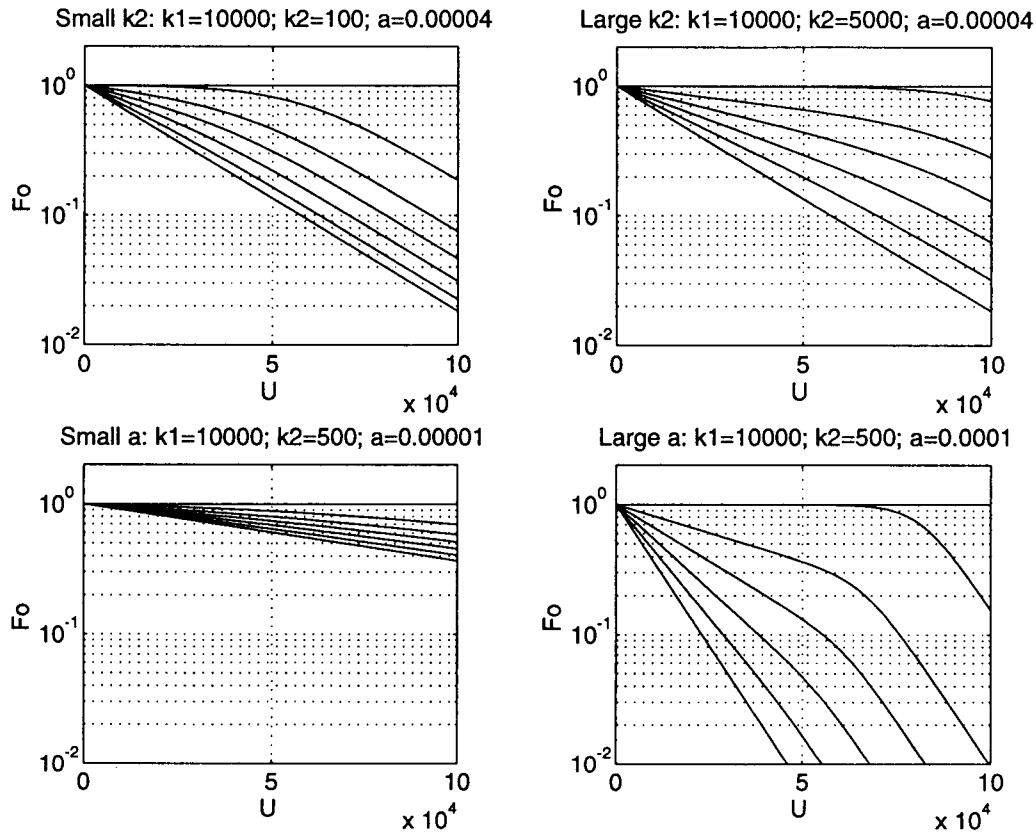

Figure 5.4: Some Mathematical Models for Different Sets of Parameters. Always $\lambda = 0$, 0.2, 0.4, 0.6, 0.8, 0.999 and 1.

CHAPTER 6. BIOLOGICAL INTERPRETATIONS

6.1 Interpretation of the Graphs

The interpretation of the graphs can only yield some qualitative information about the actual processes.

6.1.1 First Decomposition of the Graph

The first decomposition of the graph according to the decomposition shown earlier (fig. 5.1) is given in fig. 6.1.

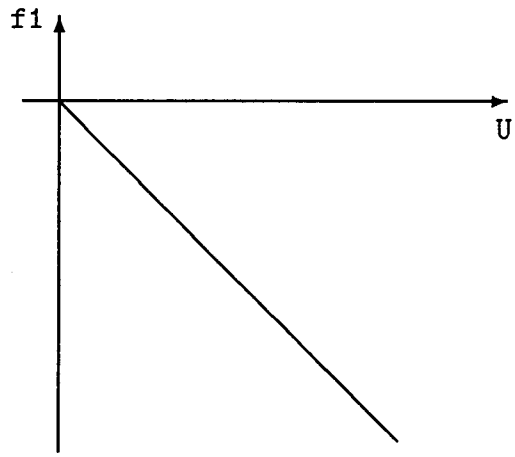

Figure 6.1: First Decomposition of the Graph of Limiting Dilution Analysis

This figure shows the well-known characteristics of single-hit kinetics. The larger the total number of lymphocytes, the larger the number of specific precursor cells, the smaller the fraction of non-responding culture wells. This

decrease occurs like in a Poisson distribution, that means it is an exponential decrease. Exponential decrease or increase occurs very often in biological systems.

6.1.2 Second Decomposition of the Graph

The second decomposition of the graph according to the decomposition shown earlier (fig. 5.1) is given in fig. 6.1.2.

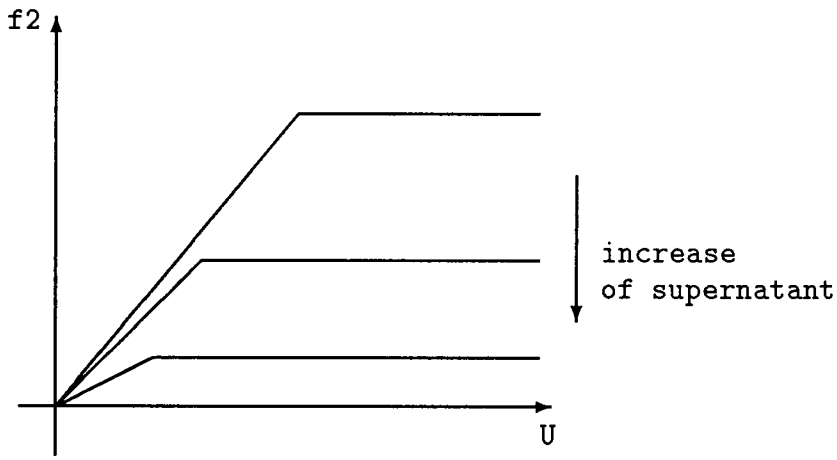

Figure 6.2: Second Decomposition of the Graph of Limiting Dilution Analysis

This graph shows an increase in the fraction of non-responding wells. This is assumed to be due to the "missing" supernatant. The larger λ is, the faster the fraction of non-responding wells decreases, since the parameter λ is a measurement for the "lack" of supernatant for a full reaction. Thus, we have an exponential increase of the fraction of non-responding culture wells due to the lack of supernatant.

This effect, however, has a saturation in U. That means that for a

certain absolute number of lymphocytes there is no further increasing effect on the fraction of non-responding wells due to $\lambda \neq 0$. Note that this absolute number of lymphocytes depends on the percentage of supernatant, λ.

At first it might seem strange that the saturation of F_0 takes place at an absolute value of U, which depends on a per capita concentration of supernatant λ. The reason for this saturation, we suspect, might be the geometry of the well.

An increase of U increases the absolute amount of supernatant (since λ is a percentage); depending on how large the fraction of supernatant is, one will reach a certain absolute amount of supernatant, which must be defined by the size and contents of the culture well, at which an increase of supernatant cannot have any further effect on F_0. This is nothing more than the saturation of the fraction of non-responding culture wells in U.

6.2 Interpretation of the Differential Equation Model

Since the interpretation of the graphs only gives a rather rough picture of the problem, we use partial differential equations to obtain a more quantitative usable idea of the occurring processes.

6.2.1 Equations

As given in eq. 5.10 in sec. 5.6 the proposed mathematical model is

$$F_0(U,\lambda) = \left(\sqrt{1+\pi(\lambda)^2}\right)^{\lambda} \cdot e^{-aU} \cdot \left(\frac{e^{aU}}{\sqrt{1+\pi(\lambda)^2 \cdot e^{2aU}}}\right)^{\lambda}. \qquad (6.1)$$

The partial derivatives with respect to U and λ, respectively, are [1]

$$\frac{\partial}{\partial U}F_0(U,\lambda) = -aF_0(U,\lambda) + a\lambda F_0(U,\lambda)\cdot\frac{1}{1+\tau^2 e^{2aU}}, \tag{6.2}$$

$$\begin{aligned}\frac{\partial}{\partial \lambda}F_0(U,\lambda) &= \frac{1}{2}\ln(1+\tau^2)\cdot F_0(U,\lambda) + \lambda\frac{\tau}{1+\tau^2}\cdot\frac{d\tau}{d\lambda}\cdot F_0(U,\lambda)\\ &\quad + aU\cdot F_0(U,\lambda) - \frac{1}{2}\ln(1+\tau^2 e^{2aU})\cdot F_0(U,\lambda)\\ &\quad - \lambda\frac{\tau e^{2aU}}{1+\tau^2 e^{2aU}}\cdot\frac{d\tau}{d\lambda}\cdot F_0(U,\lambda).\end{aligned} \tag{6.3}$$

These are partial derivatives since F_0 depends on two variables, U and λ. U and λ are independent since both are input variables, and can be chosen completely arbitrarily. Note that this is not the case for u; u depends on λ (cf. sec. 6.2.3 and sec. 6.4).

6.2.2 Way of Interpretation

It is known that the derivative of a function is the instantaneous rate of change of that function due to the change of its variable. Thus, differentiation means the study the change of a value rather than the value itself. In here we use derivatives as a way to simplify our problem of interpretation.

The full differential of a function $F_0(U,\lambda)$ is defined as

$$dF_0(U,\lambda) = \frac{\partial F_0}{\partial U}dU + \frac{\partial F_0}{\partial \lambda}d\lambda.$$

The partial derivative $\frac{\partial F_0}{\partial U}$ is the rate or velocity of change of F_0 with respect to U. It is associated with a decrease or increase of F_0, depending on the its sign, and on the type of change of U (either decrease or increase). That means a positive rate of change results for increasing U in an increase

[1] For a complete derivation of eqs. 6.2 and 6.3 see Appendix B.

of F_0, while it will decrease F_0 for decreasing U, etc.. We will use this in the interpretation of the derivative.

6.2.3 Differentiation with Respect to U

The interpretation of eq. 6.2 will be an interpretation of the components of this equation. First we will show the meaning of the equation through an example by increasing U (a decrease of U would be the other possible example), then we will state four assumptions (or hypotheses) which explain these effects, and finally we will give reasons why these assumptions are reasonable.

Note, that

$$F_0(U,\lambda) \in [0,1] \;\; \forall\, U \geq 0, \lambda \in [0,1].$$

Let us now go step by step through the equation.

EQUATION	EXAMPLE OF INCREASE OF U
$\frac{\partial}{\partial U} F_0(U,\lambda)$	The instantaneous rate of change of F_0 due to an increase of U
$=$	is
$-aF_0(U,\lambda)$	negative, which is associated with a decrease of F_0,
$+a\lambda F_0(U,\lambda)\times$	and positive depending on the "lack of supernatant" $(=\lambda)$, which is associated with an increase of F_0 depending on λ.
$\times \frac{1}{1+\tau^2 e^{2aU}}$	times a saturation effect.

The constant parameter a is present in all terms. It acts therefore like a scaling factor.

The presence of the term $F_0(U, \lambda)$ in both summands tells something about how this decrease or increase occurs. It is as fast as the instantaneous value of F_0, in other words: it is exponential.

The term $\frac{1}{1+\tau^2 e^{2aU}}$ is shown in fig. 6.3. From there it is obvious that it represents a saturation effect. For $\tau(\lambda) = [-k_2 \cdot \ln(1-\lambda)]^{-ak_1}$, the limits are

$$\lim_{\lambda \to 0} \frac{1}{1+\tau^2 e^{2aU}} = 0 \ \forall U \geq 0,$$

and

$$\lim_{\lambda \to \infty} \frac{1}{1+\tau^2 e^{2aU}} = 1 \ \forall U \geq 0.$$

This fits very good to the following assumptions, which shows that the chosen function $\tau(\lambda)$ fits good to our problem.

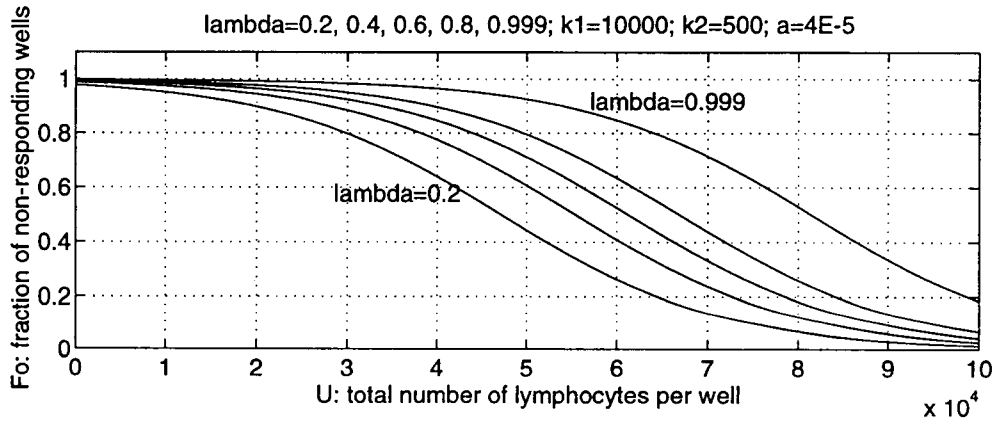

Figure 6.3: Saturation through $\frac{1}{1+\tau^2(\lambda)e^{2aU}}$ for different values of λ; $\tau(\lambda) = [-k_2 \cdot \ln(1-\lambda)]^{-ak_1}$

The assumptions for the explanation of these effects are,

1. for excess of supernatant, an increase of U increases proportionally u,

2. an increase of u decreases F_0,

3. an increase in the per capita amount of supernatant (decrease in λ) increases the per capita number of specific lymphocytes (that is the percentage associated with u),

4. there is a maximal amount of supernatant (absolute value, not percentage) when it will cease to have further effect (= saturation).

These assumptions are based on the following arguments, the

1. assumption is due to simple statistical considerations,

2. assumption is the same as in previous research,

3. assumption takes into account the changes in affinity of the lymphocytes for the antigen due to the change in supernatant,

4. assumption shows a dependence on the geometry of the culture well.

The parameter a is a rough estimate for the amount of specific precursors in the total number of lymphocytes. This is due to the fact that it does not take into account the affinity changes due to changes in supernatant concentration. It is the exact measurement only for $\lambda = 0$. Note that a does not depend on λ.

These explanations and assumptions deserve a detailed consideration.

6.2.3.1 First Assumption: Proportional Increase of U With u

This assumption implies that, in the case of excess of supernatant, u is proportional to U. If U is increased then u increases in a proportional manner.

This is obvious from statistical considerations, since U is a very large number and therefore it is very probable that we will find a certain constant percentage of specific lymphocytes in this number. However, this does not take into account the effects of the saturation as stated in assumption 4. Therefore it is true only for the case of excess of supernatant ($\lambda = 0$).

This assumption is used in the previous research as well.

6.2.3.2 Second Assumption: An Increase of u Decreases F_0

This assumption is also obvious through statistical considerations. This has been pointed out in all previous research.

If the number of specific lymphocytes (per well), u, increases, there will be more wells which respond to the antigen. Thus, the fraction of non-responding culture wells F_0 will decrease.

6.2.3.3 Third Assumption: An Increase of Supernatant Increases the Amount of Specific Precursors

This is probably the most crucial assumption. It has no equivalent in previous research. It takes into account the change of affinity of lymphocytes due to the change of supernatant concentration.

The idea is simply that less supernatant will result in less affinity. This decrease of affinity among the lymphocytes is modeled as a lower number of specific precursors u. That means that lymphocytes which would react with the antigen in the case of excess of supernatant (they would be included in u) are now counted as non-specific precursor cells (not included in u). The lower concentration of supernatant does not allow their stimulation. In the limit (no supernatant or $\lambda = 1$), there are no specific precursor cells in the culture wells

($u = 0$) for all possible amounts of U.

Note that we do not deal with this problem as one of two interacting cell types, but as a problem of one cell type reacting with the antigen. The amount of this cell type, however, depends on another factor, the supernatant concentration. Thus, this approach is different to multi-hit or multi-target kinetics as described in sec. 3.2.2.2.

Note in addition that λ describes a percentage (or concentration) of supernatant. Thus, a change of λ will have an effect on the percentage of specific precursors rather than directly on the absolute amount u. A decrease of λ decreases the *concentration* of specific precursor cells.

6.2.3.4 Fourth Assumption: Saturation of Effect of Supernatant

Saturation effects occur frequently in biological systems. In sec. 4.4 we already dealt with two other saturation effects.

This one is different to the effect mentioned in sec. 4.4.1 (excess of supernatant). With excess of supernatant we mean a maximal *concentration* of supernatant which is in our model the case of "no lack" ($\lambda = 0$). In this case we have such a large percentage of supernatant that all precursor cells which could possibly be stimulated are stimulated. There are no more possible precursors in the total number of lymphocytes. Therefore, any further increase of the supernatant concentration cannot change anything in the statistics about the non-responding wells. In this case we have the well-known single-hit kinetics as previously discussed by Lefkovits et.al. [12].

The saturation effect we deal with here has a different nature. Its occurrence depends on a certain *absolute amount* of supernatant, not on a percentage (or concentration). At this absolute amount the particular super-

natant concentration (value of λ in particular case) is not anymore important for the effect of a change of u on F_0.

The reason for this effect is that due to the fact that a culture well has a given geometry (size or volume), there will be an absolute amount of supernatant when this well is full of supernatant. When this occurs, a further increase of u will have the same effects as an increase of u in a culture well which has excess of supernatant for all amounts of U (that is the saturation effect described earlier).

The occurrence of this saturation due to an absolute amount of supernatant will depend on U. The factor λ stands for a percentage of supernatant. That means that for a constant λ with increasing U the per capita amount of supernatant does not change, however, the absolute amount increases (like U increases). Thus, the absolute amount of supernatant for which it ceases to have further effect on the change of F_0 will be obtained for a certain amount of U, called U_{knee}. U_{knee} has to depend on λ (concentration of supernatant), since the larger the concentration (or percentage), the faster is a certain absolute number achieved. This dependence is expressed through $\tau(\lambda)$.

Note that since this effect does not occur for low numbers of lymphocytes per well (small U), there will be an offset in the fraction of non-responding culture wells F_0. This offset cannot be seen in the differential equation, since it does not depend on U, but on λ. It is accounted for in the term referring to the initial condition.

6.2.3.5 Quantitative Treatment

So far we presented a rather qualitative treatment of the occurring effects. The derivative, however, treats the problem quantitatively.

We reason now, that if we can find a qualitative explanation which can fit the quantitative information given through the derivative, then we can assume that the derivative describes the quantities of the qualitative explanation. The reason for this conclusion is that the derivative fits the given data.

In other words, if we want to know in numbers what really happens, we use the derivative; if we want to know why it happens, we use the interpretations given above together with the derivative.

6.2.3.6 Interpretation of the Derivative With Respect to U

Recall the partial derivative with respect to U

$$\frac{\partial}{\partial U}F_0(U,\lambda) = -aF_0(U,\lambda) + a\lambda F_0(U,\lambda)\cdot\frac{1}{1+\tau^2 e^{2aU}}.$$

This equation deals with the effect of a change of U on F_0.

Such a change of U, e.g., increase of U, will result in an increase in u (due to assumption 1). This increase of u has to have the effect of a decrease of F_0 (assumption 2), which is the case because of the term $-aF_0(U,\lambda)$.

If $\lambda \neq 0$, the amount of specific precursor cells u in the absolute amount of lymphocytes U is diminished (due to assumption 3). This means there are less specific precursors u in a not-changing number U if λ increases. Thus, the decrease of F_0 will be diminished depending on λ. Therefore, there has to be an effect in the equation which increases F_0; this increase has to be larger, the larger λ is. This is represented in the term $a\lambda F_0(U,\lambda)$.

To fulfill assumption 4, the above mentioned effect has to have a saturation. This is the case through the term $\frac{1}{1+\tau e^{2aU}}$.

In addition, the differential equations quantify these changes. All changes

depend on $F_0(U,\lambda)$. In other words the magnitude of a change of $F_0(U,\lambda)$ depends on its instantaneous value. This kind of change is an exponential one and occurs frequently in biology.

6.2.4 Differentiation with Respect to λ

The differential equation with respect to λ is not so well understood than the one with respect to U. Especially the meaning of the logarithmic terms is difficult to grasp and since there is not enough data for a sound knowledge of $\tau(\lambda)$, it is difficult to interpret its differential. It is, however, easy to state where the terms come from in the original equation. This is given in table 6.1. Perhaps this can give some light for further interpretation.

Differential Equation	Comes From
$\frac{\partial}{\partial\lambda}F_0(U,\lambda) =$	
$\frac{1}{2}\ln(1+\tau^2)\cdot F_0(U,\lambda) + \lambda\frac{\tau}{1+\tau^2}\cdot\frac{d\tau}{d\lambda}\cdot F_0(U,\lambda)$	$(1+\tau^2)^{\frac{\lambda}{2}}$
$+aU\cdot F_0(U,\lambda) - \frac{1}{2}\ln(1+\tau^2e^{2aU})\cdot F_0(U,\lambda)$ $-\lambda\frac{\tau e^{2aU}}{1+\tau^2e^{2aU}}\cdot\frac{d\tau}{d\lambda}\cdot F_0(U,\lambda)$	$\left(\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\right)^{\lambda}$

Table 6.1: Origin of Partial Derivative with Respect to λ.

The term e^{-aU} does not have any effect on the differential equation with respect to λ other than that it is part of the factor $F_0(U,\lambda)$.

The parameter $\tau(\lambda)$ depends (besides its dependence on λ) on the geometry and possible contents of the culture well. It gives an measurement for the number of lymphocytes U needed to achieve an absolute amount of supernatant for a specific supernatant concentration λ. Thus, it gives some information when this saturation effect takes place.

6.2.4.1 The Initial Condition

The only term of the original equation which so far was not considered at all in the explanations is the term due to the initial condition.

The term which characterizes the initial condition is

$$(1 + \tau(\lambda))^{\frac{\lambda}{2}}$$

So far we cannot really explain why this term is in the equation (no such term is in the equation for single-hit kinetics; this is obvious, because in that case $\lambda = 0$). However, we do know some things about this term.

First it is nonlinearly dependent on λ (note that τ also depends on λ).

Second we know that this term comes from the saturation in U due to an absolute amount of supernatant. It can be looked at as an offset which is defined by the distance in F_0 between the curve for the single-hit kinetics ($\lambda = 0$) and the point where the single-hit kinetics-like part of the curves with $\lambda \neq 0$ starts (the location of the "knee").

This term is probably connected with the fact that one always puts the same amount of cells in a culture well — either active lymphocytes or irrigated filler cells.

6.3 Synthesis: Interpretation of the Original Equation

From our proposed model equation,

$$F_0(U, \lambda) = \left(\sqrt{1 + \tau(\lambda)^2}\right)^{\lambda} \cdot e^{-aU} \cdot \left(\frac{e^{aU}}{\sqrt{1 + \tau(\lambda)^2 \cdot e^{2aU}}}\right)^{\lambda}.$$

The two main effects, that give rise to this equation are:

(1) e^{-aU} is a decrease of $F_0(U,\lambda)$ due to an increase in u proportional to U without taking into account the effects associated with λ,

(2) $\frac{e^{a\lambda U}}{(1+\tau^2 e^{2aU})^{\frac{\lambda}{2}}}$ is the diminishing effect on u of the decrease of per capita supernatant, which results in an increase of $F_0(U,\lambda)$. The denominator is due to the fact that this diminishing effect on u has a saturation.

In addition there is a term present which we referred to as associated with the initial condition. It is

$$(1+\tau^2)^{\frac{\lambda}{2}}.$$

This factor increases F_0 overall. Thus, F_0 is overall the larger, the less per capita supernatant is present. It is called the "initial condition" since it makes sure that $F_0(0,\lambda) = 1$. This is achieved through the cancellation with the denominator of the term described in (2). It has a noticeable effect for large values of U (close to the "knee" and especially after), since then it does not cancel anymore with the denominator of this term.

This increase of $F_0(U,\lambda)$ can be expressed as a slower decrease of $F_0(U,\lambda)$ compared to the case $\lambda = 0$. The slower decrease of F_0 has the effect that for large U F_0 has a higher value for $\lambda \neq 0$ compared to $\lambda = 0$. Therefore, the factor $(1+\tau^2)^{\frac{\lambda}{2}}$ is a measurement for the difference between $F_0(U,\lambda)$ for $\lambda \neq 0$ and $F_0(U,0)$ at the point of the "knee". The amount $U = U_{knee}$ of the occurrence of the "knee" will depend on λ and on the geometry of the well (expressed through $\tau(\lambda)$).

6.4 What is u and the Frequency $f(\lambda)$?

For practical reasons it is very important to know the amount of specific precursors u and the frequency $f(\lambda) = \frac{u}{U}$. However, since in this approach we modeled the change of affinity of precursors due to λ in a change of u, it is difficult to define what we actually mean by u. If we subsequently stick to this approach (in particular to assumption 3 in sec. 6.2.3), the number of specific precursors u must be 0 if there is no supernatant ($\lambda = 1$). In that case the fraction of non-responding culture wells has to depend on u like in the case of single-hit kinetics. The reason for this is that we actually deal the problem like single-hit kinetics, with the only difference that in our case u is not independent. It depends on λ. This, however, does not change the relation between F_0 and u, it affects only the relationship between F_0 and U or u and U.

If we call in this case the fraction of non-responding wells $F_0'(u)$ (to distinguish from $F_0(U, \lambda)$, which depends on different variables), then we get

$$F_0'(u) = e^{-u}.$$

Together with our results for $F_0(U, \lambda)$ we get, since the fraction of non-responding wells is for all variables the same, i.e.,

$$F_0'(u) = e^{-u} = F_0(U, \lambda) = \left(\sqrt{1 + \tau(\lambda)^2}\right)^{\lambda} \cdot e^{-aU} \cdot \left(\frac{e^{aU}}{\sqrt{1 + \tau(\lambda)^2 \cdot e^{2aU}}}\right)^{\lambda}, \tag{6.4}$$

and

$$u = -\frac{\lambda}{2}\ln(1 + \tau^2) + aU - \lambda aU + \frac{\lambda}{2}\ln(1 + \tau^2 e^{2aU}). \tag{6.5}$$

This is a rather complicated equation and it would be nice to somehow

separate U out, especially from the last term.

A first step to understand this equation is to see where it originated from in eq. 6.4, i.e.,

$$u = \underbrace{-\frac{\lambda}{2}\ln(1+\tau^2)}_{\text{initial condition}} + \underbrace{aU}_{e^{-aU}} - \underbrace{\lambda aU}_{\text{effect of } \lambda \text{ on } F_0} + \underbrace{\frac{\lambda}{2}\ln(1+\tau^2 e^{2aU})}_{\text{saturation effect}}.$$

A second step is to look at the limiting behavior, that is, when $U \to 0$ and $U \to \infty$.

$$\begin{aligned}\lim_{U\to 0} u &= \lim_{U\to 0}\left(-\frac{\lambda}{2}\ln(1+\tau^2) + a(1-\lambda)U + \frac{\lambda}{2}\ln(1+\tau^2)\right) \\ &= \lim_{U\to 0}\left(a(1-\lambda)U\right) \quad [=0].\end{aligned}$$

Thus, close to $U = 0$ we have a frequency of

$$f_{small}(\lambda) = \frac{u}{U} = a(1-\lambda). \tag{6.6}$$

The other limit is

$$\begin{aligned}\lim_{U\to\infty} u &= \lim_{U\to\infty}\left(-\frac{\lambda}{2}\ln(1+\tau^2) + aU - a\lambda U + \frac{\lambda}{2}2aU + \frac{\lambda}{2}\ln\tau^2\right) \\ &= \lim_{U\to\infty}\left(\lambda\ln\tau - \frac{\lambda}{2}\ln(1+\tau^2) + aU\right) \quad [\to\infty].\end{aligned}$$

Therefore, for large U, the frequency is

$$f_{large}(\lambda) = \frac{u - \lambda\ln\tau + \frac{\lambda}{2}\ln(1+\tau^2)}{U} = a. \tag{6.7}$$

The term $\left[\lambda\ln\tau - \frac{\lambda}{2}\ln(1+\tau^2)\right]$ is an offset due to the saturation described in sec. 6.2.3, it is the only difference to single-hit kinetics, since $f(\lambda = 0) = a$.

CHAPTER 7. APPLICATIONS

7.1 For Interpretation

The model describes the system requirements in an exact way. Therefore, it facilitates the understanding of the biological processes which are responsible for the specified (or required) system behavior. In other words, the equation that represents our mathematical model is like a biological law described in mathematical terms. From this law we can derive new insights, like that the frequency f changes with U or that the effect on F_0 which depends on λ has a saturation in U.

These new insights are closely related to the way we interpreted the model. Therefore, it is impossible to derive, from a model like this, laws with absolute certainty. A model cannot prove such laws, but it can open our eyes for new ways of interpretation and give evidence for our conclusions.

7.2 System Identification

7.2.1 Method

To use the model for an application it is crucial to know how to fit the model to the data, or in other words, how to identify the parameters.

We suggest the following steps,

- Find a through the case $\lambda = 0$.

  In this case $F_0(U, 0) = e^{-aU}$. Therefore the slope in the semi-logarithmic

plot will be $-a$, or

$$a = \frac{\Delta \text{ordinate}}{\Delta \text{abscissa}} = -\frac{\Delta \ln F_0(U)}{\Delta U},$$

or since the curve goes through the origin:

$$a = -\frac{\ln F_0(U)}{U}. \tag{7.1}$$

The curve has a constant slope for $\lambda = 0$, therefore one can choose any value of U to calculate a with eq. 7.1.

- Find the value of λ for each data curve.

  To achieve this, use the initial slopes of the curves. For the initial linear parts of the curves (small U) we have

  $$F_0 = e^{-a(1-\lambda)U}.$$

  Thus,

  $$\begin{aligned} a(1-\lambda) &= -\frac{\ln F_0}{U}, \\ \lambda &= 1 + \frac{\ln F_0}{aU}. \end{aligned} \tag{7.2}$$

  Again, any U in the area of the initial linear part is suitable to calculate λ, however, it should be well before the "knee".

- Find U_{knee} through the data plots.

  This is most easily accomplished by drawing two straight lines in the graph:

  - prolong the curve at its initial slope in a straight line

– prolong the curve at its final slope in a straight line

These two lines intercept at the "knee". Read U_{knee} from the abscissa. Now find $\tau(\lambda)$ by applying the eq. 5.8, that is

$$\tau(\lambda) = e^{-aU_{knee}} \tag{7.3}$$

We suggest not to bother to find an equation for τ that dependents on λ. We suggest rather to find a value for $\tau(\lambda)$ for each curve and to use these values in the model.

7.2.2 Examples

7.2.2.1 A Mathematical Model

Consider the data in fig. 4.1 (see also appendix A).

- First we find a through the line for $\lambda = 0$.

  Choosing $U = 4 \cdot 10^4$ implies that $F_0(4 \cdot 10^4, 0) = 0.23$.

  Thus

$$a = -\frac{\ln 0.23}{4 \cdot 10^4} = 3.67 \cdot 10^{-5}.$$

- Now calculate λ for the supernatant concentrations of 50 % and "0" %.

50 % : $U = 2 \cdot 10^4$ implies $F_0(2 \cdot 10^4, \lambda_1) = 0.85$.

Thus

$$\lambda_1 = 1 + \frac{\ln 0.85}{3.67 \cdot 10^{-5} \cdot 2 \cdot 10^4} = 0.78.$$

"0" % : $U = 2 \cdot 10^4$ implies $F_0(2 \cdot 10^4, \lambda_2) = 0.90$.

Thus

$$\lambda_2 = 1 + \frac{\ln 0.90}{3.67 \cdot 10^{-5} \cdot 2 \cdot 10^4} = 0.86,$$

$U = 4 \cdot 10^4$ implies $F_0(4 \cdot 10^4, \lambda_2) = 0.87$,

and

$$\lambda_2 = 1 + \frac{\ln 0.87}{3.67 \cdot 10^{-5} \cdot 4 \cdot 10^4} = 0.905.$$

Choose $\lambda = 0.89$.

- To find the knee consider the lines drawn in fig. 7.1 and read from there U_{knee}.

  "0" % : $U_{knee} = 6 \cdot 10^4$ implies $\tau(\lambda = 0.89) = e^{-3.67 \cdot 10^{-5} \cdot 6 \cdot 10^4} = 0.11$.

  "50" % : $U_{knee} = 4.6 \cdot 10^4$ implies $\tau(\lambda = 0.78) = e^{-3.67 \cdot 10^{-5} \cdot 4.6 \cdot 10^4} = 0.18$.

Applying all these parameters to the mathematical model we get the result depicted in fig. 7.2. This model can be further improved by using methods like Least-Squares or others to achieve a better fit of the slopes of model and data (cf. [39, Ljung]).

Furthermore, as always, a larger set of data points will better the parameter identification.

Note, that this model does not apply the knee-function $\tau(\lambda)$ in a closed form, it rather uses single values for $\tau(\lambda)$ for different λ.

7.2.2.2 Values for λ

Consider fig. 4.4 or 7.3 (see also appendix A).

In this case it is impossible to find $\tau(\lambda)$, however, this set of data is interesting concerning the values of λ associated with different supernatant concentrations. See table 7.1.

Supernatant Concentration	Calculated at	F_0	$a = -\frac{\ln F_0}{U}$
90 %	$U = 1 \cdot 10^4$	0.68	$3.85 \cdot 10^{-5}$
90 %	$U = 2 \cdot 10^4$	0.48	$3.67 \cdot 10^{-5}$
90 %	$U = 3 \cdot 10^4$	0.32	$3.80 \cdot 10^{-5}$
90 %	$U = 4 \cdot 10^4$	0.23	$3.67 \cdot 10^{-5}$

Table 7.1: Data for 90 % .

With $a = 3.75 \cdot 10^{-5}$ we get table 7.2.

Thus, we get the following averages,

0 % supernatant: $\lambda = \frac{0.95+0.85+0.89+0.91}{4} = 0.9$

30 % supernatant: $\lambda = 0.88$

70 % supernatant: $\lambda = 0.36$

From these results we can see that 0 % supernatant is in reality just a little bit less than 30 %.

Since the data does not include more than four supernatant concentrations it is difficult to make judgements about the structure of the relationship between the supernatant concentration and λ. The results suggest however that it is a non-linear relationship.

Supernatant Concentration	Calculated at	F_0	$\lambda = 1 + \frac{\ln F_0}{aU}$
0 %	$U = 1 \cdot 10^4$	0.98	0.95
0 %	$U = 2 \cdot 10^4$	0.90	0.85
0 %	$U = 3 \cdot 10^4$	0.88	0.89
0 %	$U = 4 \cdot 10^4$	0.87	0.91
30 %	$U = 1 \cdot 10^4$	0.97	0.92
30 %	$U = 2 \cdot 10^4$	0.88	0.83
30 %	$U = 3 \cdot 10^4$	0.88	0.89
30 %	$U = 4 \cdot 10^4$	0.85	0.89
70 %	$U = 1 \cdot 10^4$	0.80	0.40
70 %	$U = 2 \cdot 10^4$	0.60	0.32
70 %	$U = 3 \cdot 10^4$	0.50	0.38
70 %	$U = 4 \cdot 10^4$	0.38	0.35

Table 7.2: Rest of the Data.

7.3 Future Perspective

For future application of this model it might be interesting to utilize more sophisticated methods of system identification as given, e.g., in [39, Ljung]. However, for practical purposes it is desired to make the system identification as simple as possible. Therefore the method presented here seems to be a suitable way of application.

Furthermore, future data will be a source of more information concerning the "knee-function" $\tau(\lambda)$. It is so far ill-defined due to the lack of sufficient data.

Finally, the application of this model is essential to give evidence for the validity of it. Together with the biological interpretations this will be the key to strengthening this approach.

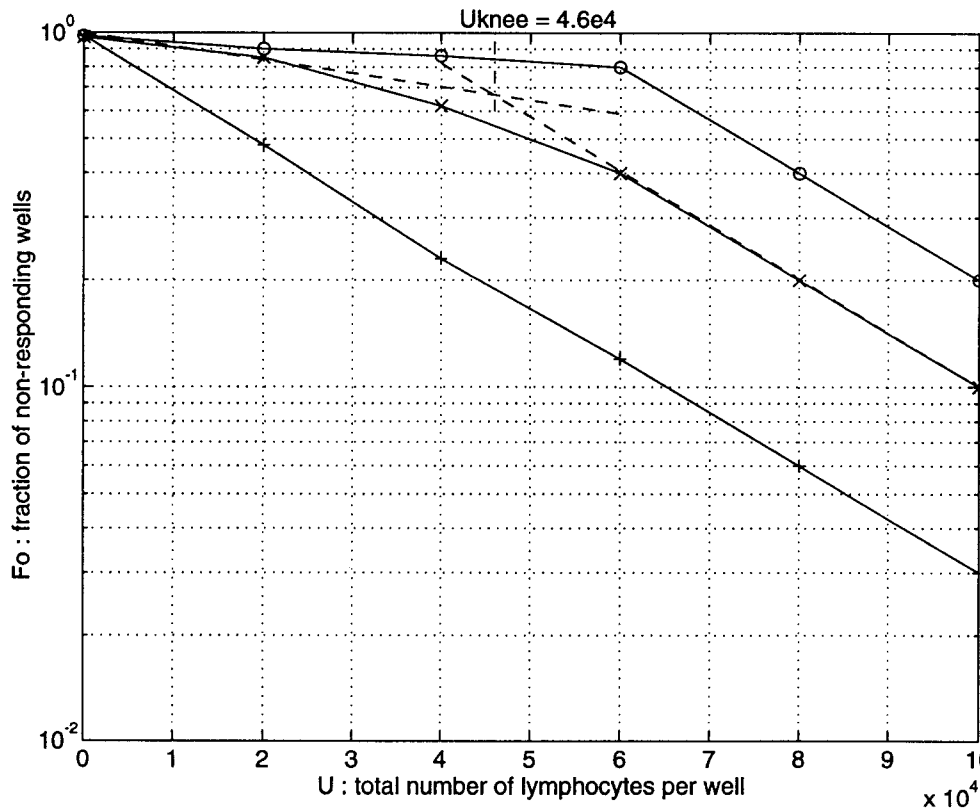

Figure 7.1: The Data from Fig. 4.1.

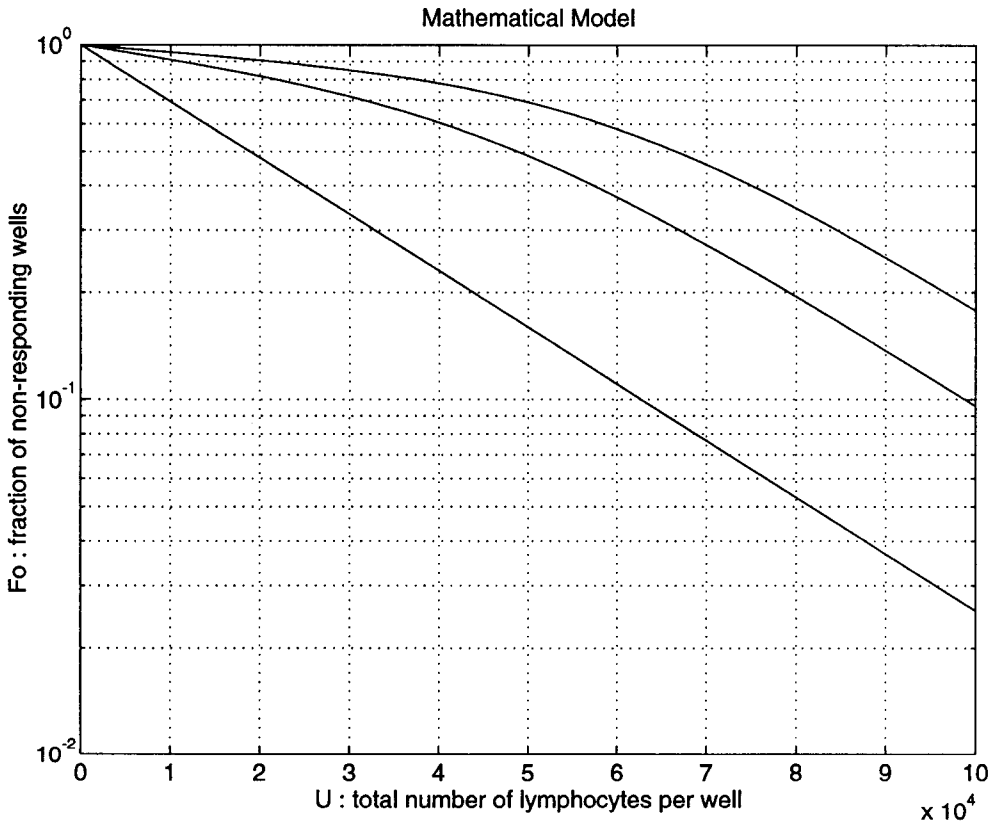

Figure 7.2: The Mathematical Model for $\lambda = 0$; $\lambda = 0.78$, $U_{knee} = 4.6 \cdot 10^4$; $\lambda = 0.89$, $U_{knee} = 6 \cdot 10^4$.

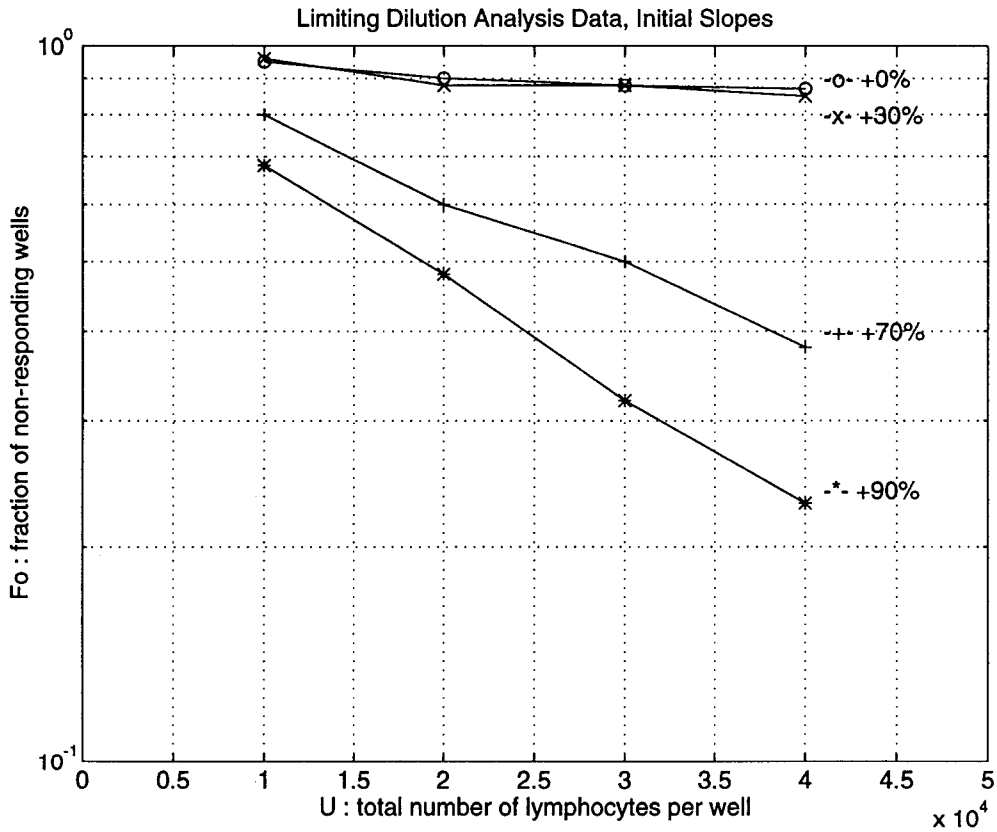

Figure 7.3: The Data Set for the Identification of Values of λ. As presented earlier.

Chapter 8. Conclusions

The model presented here yields very satisfying results in several respects. In particular

- it fits the data very well, all the requirements are fulfilled,
- it can be biologically interpreted,
- there is an easy way of applying the model (concerning system identification or the calculation of f and u).

In addition, we used some approaches which set apart this thesis from similar work. We established a mathematical model in closed form rather than an approximation using a series, and we used partial derivatives to aid the biological interpretation.

Some special features of the model interpretation are the saturation effect due to geometry and the dependence of u on λ. Especially the latter is a new way of interpretation. We deal with the system as if it depends on just one variable — u — but this variable is influenced by the supernatant concentration (represented by λ). At first this does not help to find an equation, however, it is the key for the explanation of the equations. After obtaining explanations, this approach yields ultimately a relationship between u, λ, and U. With the help of this equation the solution to the problem of modeling this process is completed. It is now possible to calculate u or to estimate the frequency f.

In another sense this thesis is very significant. In most cases mathematical equations are used as a different, exact, and formalized way of description,

or a tool for modifications or influences on a system like the control of it. In our case, however, we use mathematics as a tool for the interpretation or the understanding of the system. Essentially, you normally first understand something and then use mathematics on it, we first use mathematics and then understand. This is a different and promising approach and we hope it will find wider application.

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APPENDICES

Appendix A. The Numeric Data

In the following we include the complete numerical data as depicted in figs. 4.1-4.4. The data was provided by Mr. Henry Ortega, M.S., in the laboratory of Prof. Steven Kaattari at Oregon State University, Corvallis, Oregon. For details see sec. 3.1.

In the following, the column headings are explained.

R:F ..... Ratio of "responders" to filler cells. The "responders" are all lymphocytes capable of producing antibody, no matter if specific or not for the antigen. Thus, the "responders" are U. The filler cells are irradiated. They are incapable of producing antibody. All numbers have to be multiplied by 10^4.

Supernatant ..... The supernatant concentration added to the culture wells.

No. of Non-Resp. Wells .. Absolute amount of non-responding culture wells among 60 assayed wells with specified R:F and supernatant concentration.

Frac. of Non-Resp. Wells Fraction of non-responding culture wells F_0.

Conf. Interval ..... The 95 % confidence intervals as given in Lefkovits [12]. They are adapted from [38]. These confidence intervals are mere convention and only completely true for Poisson distributed F_0, which is the case for excess of supernatant.
Compare also sec. 4.1.

Data for Fig. 4.1

R:F in 10000	Supernatant	No. of Non-Resp. Wells	Frac. of Non-Resp. Wells	Conf. Interval
0:10	0 %	59	98 %	.91-1
2:8	0 %	54	90 %	.79-.96
4:6	0 %	52	87 %	.75-.94
6:4	0 %	48	80 %	.68-.89
8:2	0 %	24	40 %	.28-.53
10:0	0 %	12	20 %	.110-.32
0:10	50 %	58	97 %	.88-1
2:8	50 %	51	85 %	.73-.93
4:6	50 %	37	62 %	.48-.74
6:4	50 %	24	40 %	.28-.53
8:2	50 %	12	20 %	.110-.32
10:0	50 %	6	10 %	.038-.21
0:10	90 %	59	98 %	.91-1
2:8	90 %	29	48 %	.35-.62
4:6	90 %	14	23 %	.134-.36
6:4	90 %	7	12 %	.048-.23
8:2	90 %	4	7 %	.018-.16
10:0	90 %	2	3 %	.004-.115

Table A.1: Data for Fig. 4.1

Data for Fig. 4.2

R:F in 10000	Supernatant	No. of Non-Resp. Wells	Frac. of Non-Resp. Wells	Conf. Interval
0:10	0 %	59	98 %	.91-1
2:8	0 %	53	88 %	.77-.95
4:6	0 %	50	83 %	.71-.92
6:4	0 %	47	78 %	.66-.88
8:2	0 %	23	38 %	.26-.52
10:0	0 %	11	18 %	.095-.3
0:10	50 %	59	98 %	.91-1
2:8	50 %	50	83 %	.71-.92
4:6	50 %	36	60 %	.47-.72
6:4	50 %	23	38 %	.26-.52
8:2	50 %	13	22 %	.120-.34
10:0	50 %	6	10 %	.038-.21
0:10	90 %	59	98 %	.91-1
2:8	90 %	28	47 %	.34-.6
4:6	90 %	12	20 %	.11-.32
6:4	90 %	6	10 %	.038-.21
8:2	90 %	2	3 %	.004-.115
10:0	90 %	1	2 %	.001-.089

Table A.2: Data for Fig. 4.2

Data for Fig. 4.3

R:F in 10000	Supernatant	No. of Non-Resp. Wells	Frac. of Non-Resp. Wells	Conf. Interval
1:9	20 %	57	95 %	.86-.99
2:8	20 %	54	90 %	.79-.96
3:7	20 %	53	88 %	.77-.95
4:6	20 %	52	87 %	.75-.94
5:5	20 %	50	83 %	.71-.92
6:4	20 %	39	65 %	.52-.77
7:3	20 %	29	48 %	.35-.62
8:2	20 %	19	32 %	.34-.60
9:1	20 %	14	23 %	.134-.36
2:8	90 %	29	48 %	.35-.62
4:6	90 %	14	23 %	.134-.36
6:4	90 %	8	13 %	.06-.25
8:2	90 %	5	8 %	.028-18

Table A.3: Data for Fig. 4.3

Data for Fig. 4.4

R:F in 10000	Supernatant	No. of Non-Resp. Wells	Frac. of Non-Resp. Wells	Conf. Interval
1:9	0 %	57	98 %	.91-1
2:8	0 %	54	90 %	.79-.96
3:7	0 %	53	88 %	.77-.95
4:6	0 %	52	87 %	.75-.94
1:9	30 %	58	97 %	.88-1
2:8	30 %	53	88 %	.77-.95
3:7	30 %	53	88 %	.77-.95
4:6	30 %	51	85 %	.73-.93
1:9	70 %	48	80 %	.68-.89
2:8	70 %	36	60 %	.47-.72
3:7	70 %	30	50 %	.37-.63
4:6	70 %	23	38 %	.26-.52
1:9	90 %	41	68 %	.55-.8
2:8	90 %	29	48 %	.35-.62
3:7	90 %	19	32 %	.2-.45
4:6	90 %	14	23 %	.134-.36

Table A.4: Data for Fig. 4.4

Appendix B. Derivation of the Partial Differential Equations

This appendix gives the derivation of the partial differential equations eqs. 6.2 and 6.3 in sec. 6.2.

B.1 Derivative of F_0 with respect to U

Taking the partial derivative with respect to U, yields

$$\frac{\partial}{\partial U}F_0(U,\lambda) = \frac{\partial}{\partial U}\left[\left(\sqrt{1+\tau^2}\right)^{\lambda}\cdot e^{-aU}\cdot\left(\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\right)^{\lambda}\right].$$

Let

$$k(\lambda) = \left(\sqrt{1+\tau^2}\right)^{\lambda}. \tag{B.1}$$

Then

$$\begin{aligned}\frac{\partial}{\partial U}F_0(U,\lambda) &= k(\lambda)\left(\frac{\partial}{\partial U}e^{-aU}\right)\cdot\left(\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\right)^{\lambda}\\ &\quad +k(\lambda)\cdot e^{-aU}\cdot\left(\frac{\partial}{\partial U}\left(\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\right)^{\lambda}\right)\\ &= k(\lambda)(-ae^{-aU})\left(\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\right)^{\lambda}\\ &\quad +k(\lambda)\cdot e^{-aU}\cdot\lambda\left(\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\right)^{\lambda-1}\cdot\left(\frac{\partial}{\partial U}\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\right).\end{aligned} \tag{B.2}$$

Now consider

$$\begin{aligned}\frac{\partial}{\partial U}\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}} &= \frac{ae^{aU}\sqrt{1+\tau^2e^{2aU}} - e^{aU}\frac{1}{2\sqrt{1+\tau^2e^{2aU}}}\cdot 2a\tau^2e^{2aU}}{1+\tau^2e^{2aU}} \\ &= \frac{ae^{aU}(1+\tau^2e^{2aU}) - ae^{aU}\cdot\tau^2e^{2aU}}{(1+\tau^2e^{2aU})\sqrt{1+\tau^2e^{2aU}}} \\ &= a\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\cdot\frac{1}{1+\tau^2e^{2aU}}. \end{aligned} \tag{B.3}$$

Eq. B.3 in B.2 gives

$$\begin{aligned}\frac{\partial}{\partial U}F_0(U,\lambda) &= k(\lambda)(-ae^{-aU})\left(\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\right)^{\lambda} \\ &\quad +k(\lambda)\cdot e^{-aU}\cdot\lambda\left(\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\right)^{\lambda-1}\cdot a\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\cdot\frac{1}{1+\tau^2e^{2aU}} \\ \frac{\partial}{\partial U}F_0(U,\lambda) &= -aF_0(U,\lambda) + a\lambda F_0(U,\lambda)\cdot\frac{1}{1+\tau^2e^{2aU}}. \end{aligned} \tag{B.4}$$

Eq. B.4 is eq. 6.2 in sec. 6.2.

B.2 Derivative of F_0 with Respect to λ

Taking the partial derivative with respect to λ, we get

$$\frac{\partial}{\partial\lambda}F_0(U,\lambda) = \frac{\partial}{\partial\lambda}e^{-aU}\cdot\left(\sqrt{1+\tau^2}\right)^{\lambda}\cdot\left(\frac{e^{aU}}{\sqrt{1+\tau^2e^{2aU}}}\right)^{\lambda}.$$

It is known that

$$\frac{\partial}{\partial t}\ln x(t) = \frac{1}{x(t)}\frac{\partial x}{\partial t} \Rightarrow \frac{\partial x}{\partial t} = x(t)\frac{\partial}{\partial t}\ln x(t). \tag{B.5}$$

Therefore

$$\ln F_0(U,\lambda) = -aU + \frac{\lambda}{2}\ln(1+\tau^2) + \lambda aU - \frac{\lambda}{2}(1+\tau^2 e^{2aU}),$$

and

$$\frac{\partial}{\partial\lambda}(\ln F_0) = \frac{1}{2}\ln(1+\tau^2) + \frac{\lambda}{2}\frac{2\tau}{1+\tau^2}\frac{d\tau}{d\lambda} + aU - \frac{1}{2}(1+\tau^2 e^{2aU}) - \frac{\lambda}{2}\frac{2\tau e^{2aU}}{1+\tau^2 e^{2aU}}\frac{d\tau}{d\lambda}. \tag{B.6}$$

Thus, since because of eq. B.5

$$\frac{\partial}{\partial\lambda}F_0(U,\lambda) = F_0(U,\lambda)\cdot\frac{\partial}{\partial\lambda}\ln F_0(U,\lambda),$$

we get

$$\begin{aligned}\frac{\partial}{\partial\lambda}F_0(U,\lambda) &= \frac{1}{2}\ln(1+\tau^2)\cdot F_0(U,\lambda) + \lambda\frac{\tau}{1+\tau^2}\cdot\frac{d\tau}{d\lambda}\cdot F_0(U,\lambda) \\ &\quad + aU\cdot F_0(U,\lambda) - \frac{1}{2}\ln(1+\tau^2 e^{2aU})\cdot F_0(U,\lambda) \\ &\quad - \lambda\frac{\tau e^{2aU}}{1+\tau^2 e^{2aU}}\cdot\frac{d\tau}{d\lambda}\cdot F_0(U,\lambda). \end{aligned} \tag{B.7}$$

Eq. B.7 is eq. 6.3 in sec. 6.2.

Appendix C. MATLAB Scripts and Functions to Produce the Figures

This appendix gives the MATLAB scripts and functions used to produce the graph of the possible knee functions shown in fig. 5.2 and the graph of the simulated model fig. 5.3. Other graphs in this thesis were also generated using MATLAB. We did not include more scripts or functions, because only the reproduction of the two above mentioned graphs seems to be useful in the process of modeling a similar system.

All MATLAB scripts and functions were written for MATLAB Version 4.1 available in UNIX workstations.

C.1 The Knee Function

The MATLAB script to produce fig. 5.2 is

```
%script thplknee
hold off;
subplot(111)
[u,l]=thknee(10000,200);
plot(l,u)
text(.25,47000,'(10000,200)')
hold on;
grid
[u,l]=thknee(10000,50);
plot(l,u)
text(.5,32000,'(10000,50)')
```

```
[u,l]=thknee(10000,150);
plot(l,u)
text(.25,35000,'(10000,150)')
[u,l]=thknee(10000,500);
plot(l,u)
text(.5,65000,'(10000,500)')
[u,l]=thknee(10000,1500);
plot(l,u)
text(.25,70000,'(10000,1500)')
xlabel('lambda')
ylabel('uknee')
hold off
```

The MATLAB function **thknee** used in that script is

```
function [u,l]=thknee(k1,k2);
for l0=0:.01:1,
  u0=k1*log(-k2*log(1-l0));
  l=[l;l0];
  u=[u;u0];
end
```

C.2 The Mathematical Model

The MATLAB script to produce fig. 5.3 is

```
% script thplmodel.m
subplot(111)
hold off
```

```
  [f,u]=thmaster(10000,500,.999,4e-5,1000,100000);
  semilogy(u,f)
  axis([0 100000 .01 2])
hold on
for l = 0:.2:1,
  [f,u]=thmaster(10000,500,l,4e-5,1000,100000);
  semilogy(u,f)
end
xlabel('U : total number of lymphocytes per well')
ylabel('Fo : fraction of non-responding wells')
text(28000,.2,'lambda=0')
text(77000,1.2,'lambda=1')
title('lambda=0, 0.2, 0.4, 0.6, 0.8, 0.999, 1;
k1=10000; k2=500; a=0.00004')
grid
```

The MATLAB function thmaster used in the script is

```
function [f,u] = thmaster(k1,k2,l,a,ustep,umax);
% [F,u] = thmaster(k1,k2,lam,a,ustep,umax)
%  Yields the absolute value:
%  F = 1/(exp(a*u))*(sqrt(1+tau^2))^lam *
%       * ((exp(a*u))/sqrt(1+exp(2*a*u)*tau^2))^lam
%  with
%  tau = ( -k2*log(1-lam) )^(-a*k1),
%  uknee = - 1/a * log(tau),
%  and the instants of the variable u for
%  which F was computed over a u-range of
```

```
%  0 < u < umax with stepsize ustep .
%

t = (-k2*log(1-l))^(-a*k1);
if l==1, t=0; end
for u0 = 0:ustep:umax,
   f0 = exp(-a*u0)*(sqrt(1+t*t))^l *
((exp(a*u0))/(sqrt(1+exp(2*a*u0)*t*t)))^l;
   f = [f; f0];
   u = [u;u0];
end;
```

The variable `l` is λ, `t` is τ. The line `if l==1, t=0; end` was included since $\lim_{l \to 1}(-k2 \cdot log(1-l))^{-a \cdot k1} = 0$, however MATLAB gets only "`NaN`" as solution, which is not plotted.